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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Ronald C. Lundquist *et al.*

Serial No.: 09/818,921

Filed: March 27, 2001

For: FERTILE TRANSGENIC CORN PLANTS

Group Art Unit: 1638

Examiner: Ann R. Kubelik

Atty. Dkt. No.: DEKM:047USD5

CERTIFICATE OF MAILING
37 C.F.R. §1.8

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November 17, 2004
Date

Robert E. Hanson

BRIEF ON APPEAL

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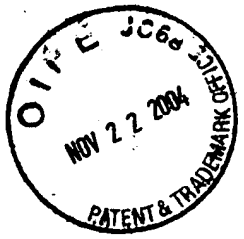

TABLE OF CONTENTS

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BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants hereby submit an original and two copies of this Appeal Brief in response to the Final Office Action dated June 16, 2004. This Brief is filed pursuant to the Notice of Appeal mailed September 14, 2004. The date for filing the instant Brief is November 17, 2004, based on the receipt of the Notice of Appeal by the Patent and Trademark Office on September 17, 2004.

The fee for filing this Appeal Brief is attached hereto. No additional fees are believed due in connection with the instant paper. However, should any other fees be due, or the attached fee be deficient or absent, the Commissioner is authorized to withdraw the appropriate fee from Fulbright & Jaworski L.L.P. Deposit Account No. 50-1212/DEKM:047USD5. Please date stamp and return the enclosed postcard to evidence receipt of this document.

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I. REAL PARTIES IN INTEREST

The real party in interest is Monsanto Company, the parent company of wholly owned subsidiary DEKALB Genetics Corporation.

II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases.

III. STATUS OF THE CLAIMS

Claims 1-9 were filed with the original specification. Claims 10-32 were added and claims 1-9 deleted in a Preliminary Amendment filed concurrently with the instant application on March 27, 2001. Claims 10-32 were pending at the time of the final Office Action and are currently pending in the case. The final rejection of claims 10-19, 21-25 and 27-32 is the subject of the instant appeal. A copy of the appealed claims prior to entry of a concurrently filed Amendment Under 37 C.F.R. § 1.116 is attached as Appendix 1. A copy of the appealed claims after entry of the Amendment is attached as Appendix 2.

IV. STATUS OF AMENDMENTS

An Amendment Under 37 C.F.R. §1.116 is being concurrently filed. The status of the Amendment is unknown.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The invention relates to a process for producing a fertile transgenic *Zea mays* plant which possesses heritable recombinant DNA that encodes a *Bacillus thuringiensis* (Bt) endotoxin, wherein the DNA is adjusted to be expressed efficiently in *Zea mays* so as to impart insect

resistance. Specification at page 7, lines 15-19. The DNA may be adjusted so that it comprises an increased number of maize preferred codons. Specification at page 18, lines 16-31. The DNA may also comprise a truncated *Bacillus thuringiensis* (Bt) endotoxin. Specification at page 20, lines 28-31

VI. ISSUES TO BE REVIEWED ON APPEAL

A. Are claims 10-19, 21-25 and 27-32 lacking enablement under 35 U.S.C. § 112, first paragraph?

B. Are claims 10-19, 21-25 and 27-32 indefinite under 35 U.S.C. § 112, second paragraph?

C. Are claims 10-19, 21-25 and 27-32 properly rejected under the judicially created doctrine of obviousness-type double patenting?

VII. ARGUMENT

A. Claims 10-19, 21-25 and 27-32 do not lack enablement

The Examiner asserts that the specification is enabling only for a method of bombarding embryogenic maize cells in the form of callus or suspension cultures that have been derived from immature embryos for the production of transformed maize plants, but not any other regenerable maize tissues. The rejection should be reversed as set forth below.

1. The rejection is without factual support

Despite having already acknowledged on the record that that claims are enabled for use of regenerable embryogenic maize cells in the form of callus and suspension cultures derived from immature embryos, the Examiner asserts that the specification is not enabling for "intact regenerable *Zea mays* cells" because specific reports culled from the prior art allegedly show

examples of failures in using certain other types of maize tissue. However, the sole evidentiary basis for this contention still relied upon in the final Office Action is the Rhodes *et al.* article, (1988, *BioTechnol* 6:56-60; **(Exhibit 1)**. This reference concerns regeneration of plants from ***protoplasts***, not ***intact regenerable Zea mays cells*** as required by the claims. It has long been known that protoplasts regenerate infertile plants, but protoplasts are ***not intact*** cells. As explained in the Merrriam Webster OnLine Dictionary™, a protoplast is "a plant cell that has had its cell wall removed." A protoplast represents the "nucleus, cytoplasm, and plasma membrane of a cell as distinguished from inert walls and inclusions." *Id.* A protoplast is therefore not intact. The Rhodes reference thus has no relevance to the enablement of the claims and the Examiner has provided no basis whatsoever to conclude otherwise.

Additional references were cited in the Office Action mailed December 24, 2003, but reliance on these appears to have been dropped by the Examiner after Applicants' response pointing out in detail the failure of these references to contradict enablement. It is therefore believed that these are no longer in issue. However, Appellants nonetheless set forth below why these references fail to cure the lack of support in the final Office Action to ensure a complete response to the rejection.

The Action dated December 24, 2003 cited Green (1975; *Crop Science* 15:417-421) **(Exhibit 2)** as allegedly showing the inability to regenerate certain cell types, such as "the failure of explants such as shoots, flowers and mature embryos to produce callus capable of regenerating whole plants." However, this reference in fact establishes success in regenerating immature maize embryos as a source of callus as of at least ***1975***. Further, given the 1975 publication date, the reference establishes a highly skilled art fifteen years before the priority date of this application. The reference demonstrates that, at best, identification of further intact

regenerable maize cells for use with the invention would require ***routine*** experimentation as those of skill in the art knew how to carry out such routine experimentation as of ***at least 1975***. Finally, it is respectfully submitted that what was not possible in 1975 is not commensurate with what was not possible as of the priority date of this application. The Green paper itself states that "[t]he ability to develop tissue and cell cultures from cereal plants has increased rapidly in recent years." **Exhibit 2**, p. 717. Further, in fields such as this where the art typically engages in experimentation, even complex experimentation would not necessarily undue. *In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd. sub nom., Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985).

A second Green reference (1982, *Proc. Fifth Internal. Cong. Plant Tiss. And Cell Cult.*, pgs. 107-108) (**Exhibit 3**) was cited by the Examiner as teaching "that embryogenic maize callus has the advantages of easy visual observation and extensive duration of regenerability in culture (pg 107, paragraphs 1, 2 and 4)." Action at p. 4, 2nd¶. The Examiner did not even attempt to contradict enablement, as the reference is cited as teaching advantages of one embodiment already acknowledged on the record to be enabled. No suggestion is made by the Examiner that it teaches the inoperability of any intact regenerable maize cells.

The Vasil reference (1987, *Theor. Appl. Genet.* 73:793-798) (**Exhibit 4**) is cited as teaching "maize's recalcitrance to regeneration in their teaching that ***protoplasts*** of an embryogenic maize culture rarely yielded callus themselves, and that while resultant callus yielded somatic embryos, they were unable to produce whole plants (p793, abstract and penultimate paragraph of column 1)." (emphasis added) Action at p. 4, 3rd¶. However, like Rhodes (1988), this reference concerns ***protoplasts***, not intact regenerable cells. It was well

known that protoplasts regenerated infertile transgenic plants but this says nothing of the ***claimed invention*** against which enablement must be analyzed.

Finally, Potrykus (1990; *BioTechnol.* 8:535-542; p540-541; (**Exhibit 5**) is cited for the proposition that "obtaining whole transformed plants requires embryogenic cells that are competent for both transformation and plant generation, in contrast to shoot meristems, as taught by Potrykus (1990, *Biotechnol.* 8:535-542; see paragraph spanning pg 540-541)." Action at p. 4, 3rd¶. However, a careful review of the section cited by the Examiner reveals that it does not say what it is claimed to say. The section does not state that shoot meristems cannot be transformed by biolistics, it merely speculates that doing so would be more difficult in cereals than in soybeans. No studies or evidence supporting this are given. This speculation and doubt is further not surprising given that the authors operate under the belief that cereal transformation had never been achieved, when in fact Applicants had done exactly that, stating that: "[w]hy then with all these advantages have no transgenic cereals been produced?" (see p540, last ¶ of **Exhibit 5**). The author even goes so far as to assert that "direct gene transfer into protoplasts should be the method of choice for those cases where such cultures have been established." Finally, the section refers to "cereal" meristems and thus it is not even clear that the comments about meristems were specific to maize, as the claims are. This reference therefore, like all of the other references cited by the Examiner, fails to negative enablement in any way.

Given the level of skill in the art, limitation of claims to intact regenerable cells, teaching in the specification, and embodiments already acknowledged on the record by the Examiner to be enabled, no basis has been provided by the Examiner to conclude that the claims are not fully enabled in compliance with 35 U.S.C. §112, first paragraph..

2. The claims are commensurate with the scope of enablement

In contradiction to the assertions of the Examiner, the claims are not directed to the use of any cells. Rather the claims are directed to use of intact regenerable cells. This subject matter has been fully enabled. The Examiner has already acknowledged that the specification enables preparation of whole, fertile transgenic maize plants using regenerable embryogenic cells in the form of callus or suspension cultures derived from immature embryos. These acknowledged examples alone are more than sufficient to establish enablement of the claims.

An assertion that the disclosure is not commensurate with the scope of the claims must be supported by evidence or reasoning substantiating the doubts advanced. *In re Dinh-Nguyen*, 181 U.S.P.Q. 46 (CCPA 1974). Enablement is satisfied as long as at least one method is provided for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claims. MPEP 2164.01(b) (citing *In re Fisher*, 427 F.2d 833, 839, 166 U.S.P.Q. 18, 24 (CCPA 1970)). The Examiner acknowledges two such embodiments fully within the scope of the claims and Appellants have further affirmatively set forth working examples demonstrating use of these embodiments.

Thus the acknowledged enabling examples alone establish enablement of the full scope of the claims is underscored by the highly advanced level of skill in the art. The references cited by the Examiner establish that ***as of at least 1975*** those of ordinary skill in the art knew how to screen different tissues for the ability to regenerate regenerating whole fertile plants. (Green (1975); **Exhibit 2**). The fact that 100% of the tissues screened may not ultimately regenerate fertile transgenic plants is irrelevant to enablement because the references cited by the Examiner show that one of skill in the art can with routine screening select tissues that are regenerable within the scope of the claims. It has never been required for enablement that every conceivable

embodiment be demonstrated to be enabled. Appellants further do not attempt to claim use of non-regenerable cells, as is apparently believed by the Examiner. The claims are therefore fully commensurate with the scope of enablement. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).

In view of the failure of the Examiner to provide any evidence supporting non-enablement; the embodiments within the scope of the claims acknowledged on the record to be enabled; the working examples and teaching in the specification and knowledge of those of skill in the art, it is submitted that the claims are fully enabled under 35 U.S.C. § 112, first paragraph. Reversal of the rejection is thus respectfully requested.

3. The rejection must be reversed under the APA

Appellants have established above that the Examiner has failed to provide facts supporting of the rejection. However, it is specifically the burden of the Examiner to do so. This is underscored by the enabled embodiments already acknowledged on the record and working examples showing use of these embodiments within the scope of the claims. Such examples may not be merely disregarded without a firm evidentiary basis for doing so. *In re Dinh-Nguyen*, 181 U.S.P.Q. 46 (CCPA 1974). This showing is further required under the Administrative Procedure Act ("APA"), which establishes that findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be supported by "substantial evidence" within the record in accordance with the 5 U.S.C. § 706(A), (E). *See Dickinson v. Zurko,* 527 U.S. 150, 50 USPQ2d 1930 (1999), *see also In re Gartside*, 203 F.3d 1305, 53 USPQ2d 1769 (Fed. Cir. 2000). The current rejections have not been supported in fact or law. The standards of the APA have therefore not been met and thus the rejection must be reversed.

In view of the foregoing, Appellants respectfully request that the rejection under 35 U.S.C. §112, first paragraph, be reversed.

B. The Claims Are Not Indefinite Under 35 U.S.C. §112, Second Paragraph

The Examiner has rejected claims 10-19, 21-25 and 27-32 under 35 U.S.C §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter Applicants regard as the invention. Appellants respond to each rejection below.

(1) The Examiner alleges that claim 10 is indefinite because step (ii) only requires identifying the preselected DNA while in step (iii) DNA that comprises the preselected DNA is expressed. Appellants note in response that the claim has been amended as follows in the concurrently-filed Amendment Under 37 C.F.R. §1.116:

> 10. (Currently amended) A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles; wherein said DNA comprises a preselected DNA sequence encoding a *Bacillus thuringiensis* endotoxin, wherein the preselected DNA sequence is adjusted to be more efficiently expressed in *Zea mays* than the native *B. thuringiensis* DNA sequence encoding said endotoxin; (ii) identifying a population of transformed cells comprising said preselected DNA sequence; and (iii) regenerating a fertile transgenic plant therefrom, wherein ~~said DNA comprising~~ said preselected DNA sequence is expressed so as to impart insect resistance to said transgenic plant and is heritable.

It is submitted that the rejection is now moot in light of the amendment.

(2) The Examiner alleges that claim 25 lacks antecedent basis for "the DNA encoding said endotoxin." Appellants note in response that the claim has been amended as follows in the concurrently-filed Amendment Under 37 C.F.R. §1.116:

25. (Currently amended) The process of claim 10, 11, 16 or 17, wherein the preselected DNA sequence encoding said endotoxin comprises an increased number of maize preferred codons compared to native *Bacillus thuringiensis* endotoxin.

It is submitted that the rejection is now moot in light of the amendment.

(3) The Examiner alleges that claim 21 is unclear regarding the preselected DNA sequence comprising a selectable marker gene. In particular, it is stated that it is unclear whether the process of claim 21 is one in which the preselected DNA comprises a selectable marker gene or if it further comprises a reporter gene.

In response, Appellants note that claim 21 further defines the selectable marker gene recited in claim 21 as conferring resistance to a specific set of herbicides. The claim reads as follows:

21. (Previously presented) The process of claim 11, wherein the selectable marker gene confers resistance or tolerance to a compound selected from the group consisting of hygromycin, sethoxydim, haloxyfop, glyphosate, methotrexate, imidazoline, sufolnylurea, triazolopyrimidine, s-triazine, bromoxynil, phosphinothricin, kanamycin, G418, 2,2-dichloropropionic acid and neomycin.

As can be seen, the claim does not require that the preselected DNA does or does not have a reporter gene. It merely further defines the selectable marker gene. It is completely irrelevant to definiteness whether a marker gene is present or not, as parent claim 11 is in the alternative and leaves this option open, as does claim 21. Otherwise, the parent claim 11 would be indefinite for maintaining this option as well. Breadth is not indefiniteness and alternative expressions are an accepted claim format. Reversal of the rejection is thus respectfully requested.

(4) The Examiner asserts that claims 28-29 lack antecedent basis for "the preselected DNA" in line 1.

Appellants note in response that the claims have been amended to insert "sequence" after "preselected DNA" in the concurrently-filed Amendment Under 37 C.F.R. §1.116. It is believed that the rejection is moot in light of the amendment.

(5) The Examiner asserts that claim 29 lacks antecedent basis for "the DNA encoding the endotoxin."

Appellants note in response that the claim has been amended as follows in the concurrently-filed Amendment Under 37 C.F.R. §1.116:

> 29. (Currently amended) The process of claim 19, wherein the preselected DNA further comprises the maize AdhIS first intron or the maize *Shrunken-2* first intron positioned between the promoter and the preselected DNA sequence encoding said endotoxin.

It is submitted that the rejection is now moot in light of the amendment.

(6) The Examiner alleges that claim 30 is unclear regarding the location of the promoter relative to the DNA sequence encoding the endotoxin and to the promoter recited in claim 19. Appellants note in response that the claim is fully definite as written. Specifically, the claim reads as follows:

> 30. (Previously presented) The process of claim 19, wherein the preselected DNA sequence further comprises a manopine synthase promoter, a nopaline synthase promoter or an octopine synthase promoter operably linked to said preselected DNA sequence.

As can be seen, the claim requires that the promoter be "***operably linked*** to said preselected DNA sequence" (emphasis added). This constitutes a concrete structural limitation defining the location of the promoter. It is well known and fundamental to molecular biology how to

operably link a promoter to any given coding sequence to obtain expression of a coding sequence. This is the general premise upon which all transgenic organisms operate. This places physical constraints on the location of the promoter, but such constraints are well known to those of skill in the art. The claim therefore fully defines the location of the promoter and there is no basis under the second paragraph of §112 to include any further structural limitation on the claim. Reversal of the rejection is thus respectfully requested.

(7) The Examiner asserts that in claim 32 it is unclear if any members of the population of plants comprise the preselected DNA sequence. Appellants note in response that the claim has been amended as follows in the concurrently-filed Amendment Under 37 C.F.R. §1.116:

> 32. (Currently amended) A population of plants obtained by breeding the transgenic plants of claim 10 wherein the preselected DNA sequence from said transgenic plant is transmitted by Mendelian inheritance through both male and female parent plants <u>to the population of plants</u>.

It is submitted that the rejection is now moot in light of the amendment.

In view of the foregoing, reversal of the rejections under 35 U.S.C. §112, second paragraph, is respectfully requested.

C. <u>The Rejection Under the Judicially-Created Doctrine of Obviousness-Type Double Patenting is Moot</u>

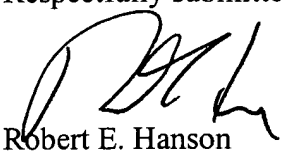
The Examiner rejected claim 32 and claims 11-32 in two separate rejections over claims 1-6 of U.S. Patent No. 5,484,956 in view of Adang *et al.* (US 5,380,831, filed September 1988) and over claims 50-51 and 57-58 of U.S. Patent No. 5,484,956, respectively. In Appellants last response, an offer was made to submit a terminal disclaimer over U.S. Patent No. 5,484,956 in

order to obviate the rejection upon an indication that the case is otherwise allowable. Appellants have therefore not further addressed the rejections in this Brief as it is believed that the rejections are now moot.

VIII. CONCLUSION

It is respectfully submitted that, in light of the above, none of the pending claims are properly rejected under 35 U.S.C. §112 first or second paragraph. Therefore, Appellants request that the Board reverse the pending grounds for rejection.

Respectfully submitted,



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Date: November 17, 2004

APPENDIX 1: LISTING OF APPEALED CLAIMS PRIOR TO ENTRY OF THE AMENDMENT UNDER 37 C.F.R. §1.116

1.-9. (Canceled)

10. (Previously presented) A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles; wherein said DNA comprises a preselected DNA sequence encoding a *Bacillus thuringiensis* endotoxin, wherein the preselected DNA sequence is adjusted to be more efficiently expressed in *Zea mays* than the native *B. thuringiensis* DNA sequence encoding said endotoxin; (ii) identifying a population of transformed cells comprising said preselected DNA sequence; and (iii) regenerating a fertile transgenic plant therefrom, wherein said DNA comprising said preselected DNA sequence is expressed so as to impart insect resistance to said transgenic plant and is heritable.

11. (Previously presented) The process of claim 10, wherein the preselected DNA sequence further comprises a selectable marker gene or a reporter gene.

12. (Previously presented) The process of claim 10 or 11, wherein the fertile transgenic *Zea mays* plant is generated from transformed embryogenic tissue.

13. (Previously presented) The process of claim 12, wherein the cells are derived from immature embryos.

14. (Previously presented) The process of claim 10 or 11, further comprising obtaining transgenic insect resistant progeny plants of subsequent generations from said fertile transgenic plant.

15. (Previously presented) The process of claim 14, further comprising obtaining seed from one of said progeny plants.

16. (Previously presented) The process of claim 10 or 11, wherein the preselected DNA sequence encodes the HD73 endotoxin of *Bacillus thuringiensis.*

17. (Previously presented) The process of claim 10 or 11, wherein the preselected DNA sequence encodes the HD1 endotoxin of *Bacillus thuringiensis.*

18. (Previously presented) The process of claim 10 or 11, wherein the preselected DNA sequence encodes the DH1 endotoxin of *Bacillus thuringiensis*.

19. (Previously presented) The process of claim 10 or 11, wherein the preselected DNA sequence comprises an operably linked promoter.

20. (Previously presented) The process of claim 19, wherein the preselected DNA sequence further comprises a promoter operably linked to said DNA sequence encoding said endotoxin and a promoter operably linked to said selectable marker gene of claim 11.

21. (Previously presented) The process of claim 11, wherein the selectable marker gene confers resistance or tolerance to a compound selected from the group consisting of hygromycin, sethoxydim, haloxyfop, glyphosate, methotrexate, imidazoline, sufolnylurea, triazolopyrimidine, s-triazine, bromoxynil, phosphinothricin, kanamycin, G418, 2,2-dichloropropionic acid and neomycin.

22. (Previously presented) The process of claim 21, wherein the compound is phosphinothricin.

23. (Previously presented) The process of claim 21, wherein the compound is kanamycin.

24. (Previously presented) The process of claim 21, wherein the compound is hygromycin.

25. (Previously presented) The process of claim 10, 11, 16 or 17, wherein the DNA encoding said endotoxin comprises an increased number of maize preferred codons compared to native *Bacillus thuringiensis* endotoxin.

26. (Previously presented) The process of claim 11, wherein the DNA encoding the *Bacillus thuringiensis* endotoxin of claim 10 is fused in frame with said selectable marker or reporter gene.

27. (Previously presented) The process of claim 18, wherein the *Bacillus thuringiensis* endotoxin comprises about the N-terminal 50% of the endotoxin.

28. (Previously presented) The process of claim 10, wherein the preselected DNA further comprises a protease inhibitor.

29. (Previously presented) The process of claim 19, wherein the preselected DNA further comprises the maize AdhIS first intron or the maize *Shrunken-2* first intron positioned between the promoter and the DNA encoding said endotoxin.

30. (Previously presented) The process of claim 19, wherein the preselected DNA sequence further comprises a manopine synthase promoter, a nopaline synthase promoter or an octopine synthase promoter operably linked to said preselected DNA sequence.

31. (Previously presented) The process of claim 19, wherein the promoter is the CaMV 35S or 19S promoter.

32. (Previously presented) A population of plants obtained by breeding the transgenic plants of claim 10 wherein the preselected DNA sequence from said transgenic plant is transmitted by Mendelian inheritance through both male and female parent plants.

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APPENDIX 2: LISTING OF APPEALED CLAIMS FOLLOWING ENTRY OF THE AMENDMENT UNDER 37 C.F.R. §1.116

1.-9. (Canceled)

10. (Currently amended) A process for producing a fertile transgenic *Zea mays* plant comprising the steps of (i) bombarding intact regenerable *Zea mays* cells with DNA-coated microprojectiles; wherein said DNA comprises a preselected DNA sequence encoding a *Bacillus thuringiensis* endotoxin, wherein the preselected DNA sequence is adjusted to be more efficiently expressed in *Zea mays* than the native *B. thuringiensis* DNA sequence encoding said endotoxin; (ii) identifying a population of transformed cells comprising said preselected DNA sequence; and (iii) regenerating a fertile transgenic plant therefrom, wherein ~~said DNA comprising~~ said preselected DNA sequence is expressed so as to impart insect resistance to said transgenic plant and is heritable.

11. (Previously presented) The process of claim 10, wherein the preselected DNA sequence further comprises a selectable marker gene or a reporter gene.

12. (Previously presented) The process of claim 10 or 11, wherein the fertile transgenic *Zea mays* plant is generated from transformed embryogenic tissue.

13. (Previously presented) The process of claim 12, wherein the cells are derived from immature embryos.

14. (Previously presented) The process of claim 10 or 11, further comprising obtaining transgenic insect resistant progeny plants of subsequent generations from said fertile transgenic plant.

15. (Previously presented) The process of claim 14, further comprising obtaining seed from one of said progeny plants.

16. (Previously presented) The process of claim 10 or 11, wherein the preselected DNA sequence encodes the HD73 endotoxin of *Bacillus thuringiensis.*

17. (Previously presented) The process of claim 10 or 11, wherein the preselected DNA sequence encodes the HD1 endotoxin of *Bacillus thuringiensis.*

18. (Previously presented) The process of claim 10 or 11, wherein the preselected DNA sequence encodes the DH1 endotoxin of *Bacillus thuringiensis*.

19. (Previously presented) The process of claim 10 or 11, wherein the preselected DNA sequence comprises an operably linked promoter.

20. (Previously presented) The process of claim 19, wherein the preselected DNA sequence further comprises a promoter operably linked to said DNA sequence encoding said endotoxin and a promoter operably linked to said selectable marker gene of claim 11.

21. (Previously presented) The process of claim 11, wherein the selectable marker gene confers resistance or tolerance to a compound selected from the group consisting of hygromycin, sethoxydim, haloxyfop, glyphosate, methotrexate, imidazoline, sufolnylurea, triazolopyrimidine, s-triazine, bromoxynil, phosphinothricin, kanamycin, G418, 2,2-dichloropropionic acid and neomycin.

22. (Previously presented) The process of claim 21, wherein the compound is phosphinothricin.

23. (Previously presented) The process of claim 21, wherein the compound is kanamycin.

24. (Previously presented) The process of claim 21, wherein the compound is hygromycin.

25. (Currently amended) The process of claim 10, 11, 16 or 17, wherein the preselected DNA sequence encoding said endotoxin comprises an increased number of maize preferred codons compared to native *Bacillus thuringiensis* endotoxin.

26. (Previously presented) The process of claim 11, wherein the DNA encoding the *Bacillus thuringiensis* endotoxin of claim 10 is fused in frame with said selectable marker or reporter gene.

27. (Previously presented) The process of claim 18, wherein the *Bacillus thuringiensis* endotoxin comprises about the N-terminal 50% of the endotoxin.

28. (Currently amended) The process of claim 10, wherein the preselected DNA sequence further comprises a protease inhibitor.

29. (Currently amended) The process of claim 19, wherein the preselected DNA sequence further comprises the maize AdhIS first intron or the maize *Shrunken-2* first intron positioned between the promoter and the preselected DNA sequence encoding said endotoxin.

30. (Previously presented) The process of claim 19, wherein the preselected DNA sequence further comprises a manopine synthase promoter, a nopaline synthase promoter or an octopine synthase promoter operably linked to said preselected DNA sequence.

31. (Previously presented) The process of claim 19, wherein the promoter is the CaMV 35S or 19S promoter.

32. (Currently amended) A population of plants obtained by breeding the transgenic plants of claim 10 wherein the preselected DNA sequence from said transgenic plant is transmitted by Mendelian inheritance through both male and female parent plants to the population of plants.

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NOV 22 2004
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APPENDIX 3

EVIDENCE APPENDIX

Exhibit 1. Rhodes *et al.* (*BioTechnol.* 6:56-60, 1988): Cited in Office Action dated December 24, 2003.

Exhibit 2. Green (*Crop Science* 15:417-421, 1975): Cited in Office Action dated December 24, 2003.

Exhibit 3. Green (*Proc. Fifth Internal. Cong. Plant Tiss. And Cell Cult.*, pgs. 107-108, 1982): Cited in Office Action dated December 24, 2003.

Exhibit 4. Vasil (*Theor. Appl. Genet.* 73:793-798, 1987): Cited in Office Action dated December 24, 2003.

Exhibit 5. Potrykus (*BioTechnol.* 8:535-542, 1990): Cited in Office Action dated December 24, 2003.

PLANT REGENERATION FROM PROTOPLASTS ISOLATED FROM EMBRYOGENIC MAIZE CELL CULTURES

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Genetic engineering of maize via protoplast technology has been limited due to lack of plant regeneration from maize protoplasts. We have developed a system of protoplast culture that results in high plating efficiency from embryogenic protoplasts and can be followed by plant regeneration. Maize protoplasts were grown on filters directly over a feeder layer of nurse cells in liquid medium. Initial condition and subsequent growth of feeder cells were critical in obtaining a plating efficiency of 10% from protoplasts. Protoplasts were digested from embryogenic cell suspension cultures, and recovered callus retained the morphogenic potential of initial donor cultures. The system has been successfully used with protoplasts of two maize inbreds, one of which is an important commercial line.

Many genetic manipulations, such as somatic hybridization, cytoplasmic recombination, and direct DNA uptake, would be possible with maize if plants could be regenerated from protoplasts. Such regeneration has been achieved with many dicot species[1,2] but with only a few Gramineae[3-6], and more recently with the important crop, rice[7-10]. Embryogenic cell suspension cultures have been the source of protoplasts in several of these reports of cereal protoplast culture[6]. While maize protoplasts have been successfully grown to callus[11-16], these protoplast-derived calli have not been capable of plant regeneration. In some cases, the donor cells were not totipotent before any protoplast manipulations[11-13], while in other cases, totipotency was partially or completely lost during the procedure of isolating and culturing protoplasts[14-16].

High rates of callus formation from protoplasts are necessary to enable efficient recovery of cell lines transformed by direct DNA uptake. Previous work with maize protoplasts and cells[13,17,18] has indicated that nurse cultures may be critical in achieving high plating efficiencies. Ludwig et al.[13] obtained 100- to 1000-fold higher plating efficiency from Black Mexican Sweet (BMS) maize protoplasts plated over feeder cells than when grown submersed in liquid medium.

In this study, several methods of constructing feeder layers were compared in order to identify a system that both promotes protoplast growth and is easy to assemble. Several parameters involving feeder layers were optimized, as well as other variables such as medium components. We have developed a culture system for maize protoplasts that can result in plating efficiencies of 10% or more. The protoplast-derived cell lines retained the same morphogenic capabilities as the original donor suspension cultures, including the ability to regenerate plants. We demonstrated that this system works well with protoplasts of 2 maize inbreds, A188 and commercially important B73.

RESULTS

The A188 and B73-1 suspension cultures, both capable of plant regeneration, consisted of several types of cell clumps: small groups of cytoplasmically dense cells (less than 100 cells per group) with no distinct epidermal layer (Fig. 1a); larger groups of small dense cells (100 to several thousands) with cells on the perimeter forming a smooth epidermal-like layer; and clumps of cells with irregular shape and of various sizes protruding from the surface of the clumps. We did not determine the developmental relationships between cell types or if a particular type of cell group was correlated with plant regeneration. The B73-2 culture, which would form somatic embryos that did not grow into plants, consisted primarily of small groups of cells with dense cytoplasms, similar to Figure 1a. Cells from this type of group (Fig. 1a) were digested most readily to protoplasts. All 3 cell cultures (A188, B73-1, and B73-2) produced similar results during protoplast isolation and culture. Except as noted, the following descriptions apply to these 3 cell cultures. Some experiments to optimize growth conditions were done only with A188 and/or B73-2.

Typical protoplast yields, when prepared as described, were between 1 and 5 × 10^6 protoplasts per gram of suspension cells (Fig. 1b). Greater than 95% of these were viable, as determined by staining with fluorescein diacetate. The average size of protoplasts from A188, B73-1, and B73-2 cultures was 20 microns in diameter. Larger protoplasts were often multinucleate, indicating fusion had occurred during protoplast isolation. Preparations of washed protoplasts contained undigested cells at frequencies from 0.5% to 5%. These cells were elongated, had thick cell walls, and contained large vacuoles.

Divisions of cells derived from protoplasts were clearly seen after 4 days of culture. Individual colonies consisting of as many as 10 to 15 cells only 7 days after protoplast isolation (Fig. 1c). After an additional 7 days on medium with lower oxmolarity and new feeder cells, individual colonies were between 100 and 500 microns in diameter. At higher protoplast plating densities, protoplast-derived calli grew as a continuous layer on the filters (Fig. 1d). Frequency of callus formation in different experiments ranged from less than 1% to more than 10% of cultured protoplasts, depending in part on the particular batch of

protoplasts. Growth rate of the donor cell cultures varied over time, despite constant growth conditions. Faster-growing cultures generally yielded more protoplasts per gram with higher plating efficiencies (10% or more) than slower-growing cultures. Other factors affecting plating efficiency are discussed below. In this study, plating efficiency was calculated as the number of calli which were 300 microns or larger by the 19th day after protoplast isolation, divided by the number of plated protoplasts.

Plating efficiency of protoplasts was 2 to 5-fold higher than the frequency of undigested cells among the protoplasts in several experiments, which provided evidence that calli were growing from protoplasts and not merely from undigested cells. Direct observations never detected any cell divisions of undigested cells among protoplasts.

Feeder layer system. Three methods of constructing feeder systems, with several cell densities, were compared for their effect on frequency of callus formation. Generally, plating efficiency of protoplasts increased as BMS feeder cell density increased. Protoplasts responded better when grown with feeder cells (in liquid or agarose medium) spread over the entire plate surface than when feeder cells were placed only directly beneath the membrane at equivalent cell densities (Fig. 2). Absorption of liquid medium surrounding the protoplasts was faster with progressively higher feeder cell densities. It also occurred faster with agarose-embedded feeders than with feeders in liquid medium. This observation may explain why optimal cell density for agarose-embedded feeders (0.7 g/plate) was lower than for liquid spread feeders (1.0 g/plate). Protoplasts had 100-fold lower plating efficiency when grown submersed in liquid or agarose-solidified medium without feeder cells (data not shown). Conditioned medium (grown with BMS cells for 3 days) could not substitute for live feeder cells (data not shown).

When cellulose pads were placed between the protoplast support filter and BMS feeder cells, protoplast plating efficiency decreased (data not shown). Distributions of callus formation from protoplasts on top of filters often corresponded to the distribution of feeder cells underneath the filters. Both of these observations indicated that proximity of feeder cells to protoplasts is beneficial.

Experiments were done to determine the optimum length of time to culture protoplasts over feeder cells. B73-2 protoplasts had the highest plating efficiency when grown over BMS feeder layers for 7 days, rather than shorter or longer periods, before transferring them to fresh medium with lower osmolarity (Fig. 3). Attempts to precondition medium by assembling feeder layers from 1 to 4 days prior to addition of protoplasts did not change protoplast response compared to using freshly made feeder layers (data not shown). Transfer of protoplasts to fresh BMS feeder cells during the second week of culture (secondary feeders) doubled the plating efficiency of B73-2 protoplasts compared to treatments with no secondary feeders (Fig. 3). Some protoplasts formed cell walls and undergone at least 4 cell division cycles by 7 days, while others divided only once. Secondary BMS feeders may be essential for promoting further division in slower-dividing cell clumps.

When B73-2 protoplasts were plated at 5 densities between 5×10^3 and 1×10^5 per filter, plating efficiency was independent of protoplast density (data not shown). This density-independent response indicates the effectiveness of this feeder system.

Growth medium and support filters. Of 3 media tested, the highest protoplast plating efficiency and the most vigorous feeder cell growth occurred on N6ap. N6ap had a large positive effect on protoplast growth during both the first and second week of culture for B73-2 (Fig. 4) and A188 protoplasts (data not shown). Protoplasts grown on MS medium consistently had lower plating efficiencies than on N6ap medium, while protoplasts on NN medium produced much less growth than on either N6ap or MS (Fig. 4). Plating efficiencies of A188 protoplasts cultured

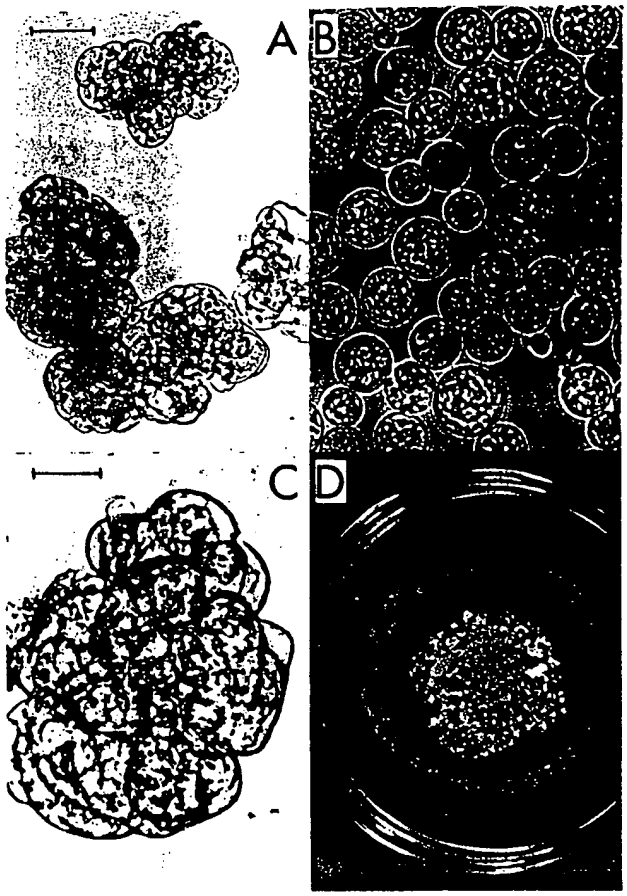

FIGURE 1. Culture of maize protoplasts. (a) groups of cells in suspension culture of A188. bar = 100 microns. (b) A188 protoplasts after isolation. bar = 20 microns. (c) callus colony 7 days after protoplast isolation. bar = 50 microns. (d) callus grown on filter (47 mm diameter) over feeder layer, 21 days after protoplast isolation.

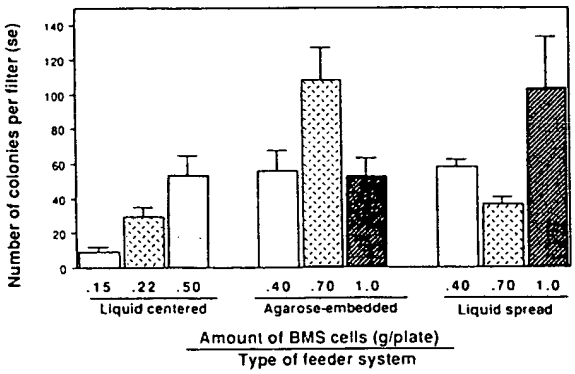

FIGURE 2. Effect of feeder system and feeder cell density on callus formation of B73 protoplasts. B73-2 protoplasts were cultured on Millipore filters (1×10^4 protoplasts/filter) in N6ap medium over BMS cells at the indicated densities. Feeder cell densities that are equivalent in g cells per unit area are indicated by identically shaded bars. Construction of feeder systems is described in Experimental Protocol. Callus colonies were counted 19 days after protoplast isolation.

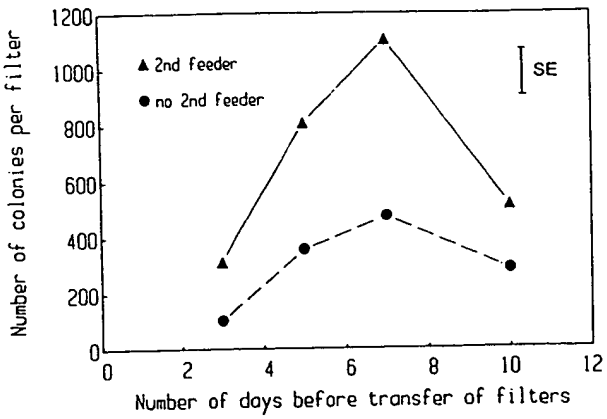

FIGURE 3. Effect of transfer timing and secondary feeder layer on plating efficiency of B73 protoplasts. B73-2 protoplasts were grown on Millipore and Nuclepore filters (5×10^4 protoplasts/filter) over BMS feeder cells embedded in agarose. At the indicated time, filters (3 replicates per treatment) were transferred to fresh N6ap medium with or without fresh BMS feeder cells. Seven days after this first transfer, filters were moved to fresh medium without feeder cells. Colonies were counted 19 days after protoplast isolation.

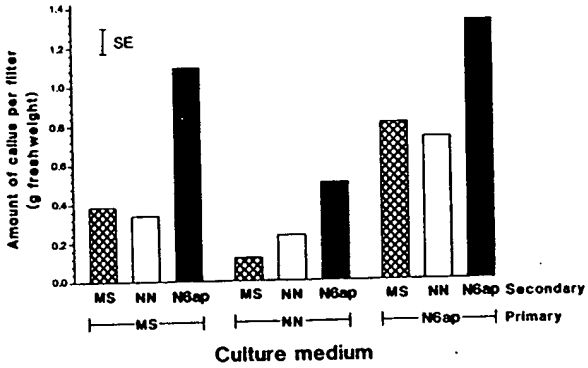

FIGURE 4. Effect of culture media on growth of B73 protoplasts. B73-2 protoplasts were grown on Millipore filters over BMS feeder cells embedded in the specified primary medium for 7 days, then transferred to secondary medium with new feeder cells for another 7 days. MS = MS with 1 mM asparagine[25]; NN = NN67[29] with 3 mM NH_4NO_3; N6ap = N6[22] with 6 mM asparagine and 12 mM proline[25]. All media contained 3% sucrose, 2 mg/l of 2,4-D, and sufficient mannitol to bring the osmolarity to 450 milliosmoles. There were 4 replicate plates per treatment with 2×10^5 protoplasts per plate.

FIGURE 5. Growth of somatic embryos from B73 protoplasts. B73-2 protoplasts were grown over BMS feeder layers in N6ap medium and transferred to fresh medium and feeders after 7 days. This photograph was taken 15 days after protoplast isolation, bar = 500 microns.

in these 3 media without feeder cells reflected the same relative ranking of media (N6ap>MS>NN), which indicates that protoplast response to these media was not solely determined by the media preference of BMS feeder cells.

The medium in which protoplasts were suspended drained through the support filter within minutes after application, when placed over feeder cells suspended in liquid medium. This effectively immobilized the protoplasts, eliminating any possibility of protoplasts spilling over the filter edge. If feeder cells were mixed with protoplasts on the filter, the genotype of individual callus could be determined by morphology: BMS cells grew as small, unorganized clumps of cells, while A188, B73-1, and B73-2 calli consisted of smaller cells in larger, organized groups, including structures resembling somatic embryos.

Several types of membranes were used to support protoplasts over feeder layers. Each type offered particular advantages involving manipulation and observation of protoplast growth, as well as having characteristics associated with their composition and pore size. The highest plating efficiency of B73-2 and A188 protoplasts (data not shown) was obtained on MSI nylon filters (E02TF04700), which are physically strong and can be reused after cleaning. Polycarbonate Nuclepore filters (8-micron-pore) (28158-840) are transparent, which permitted microscopic viewing of protoplast development *in situ*. Protoplasts were easiest to spread uniformly on Millipore filters (made of cellulose acetate and cellulose nitrate) with a 3 mm hydrophobic edge (HAEP 047 SW). Callus was most readily observed on black Millipore filters (AABP 047 SO). It took up to 4 days for medium around the protoplasts to soak through the Metricel filters (64830 TCM 450), as these filters did not contain a wetting agent. Therefore, exchange of soluble factors between feeder cells and protoplasts through these filters was delayed compared to other filters.

Plant regeneration. Callus derived from embryogenic protoplasts was capable of the same type of organized growth (somatic embryos or plant regeneration) as each of the 3 donor cell cultures. Figure 5 shows somatic embryos among less organized callus, only 15 days after protoplast isolation from B73-2 cells. Media modifications such as lower 2,4-D concentrations (0.5 and 0.1 mg/l) and higher sucrose levels (6%) during the second week of protoplast culture increased the frequency of somatic embryos among undifferentiated callus.

The B73-2 cell line that did not regenerate plants did not acquire totipotency after passage through protoplast manipulations. The A188 and B73-1 cell lines, which had the capability to regenerate plants, both retained this ability in protoplast-derived callus (Fig. 6). Eighteen percent of the protoplast-derived A188 cell lines regenerated plants as readily as the original donor culture. The remaining A188 cultures displayed the morphology of embryogenic callus, but the somatic embryos did not develop further. Thirty-two A188 cell lines from 6 different experiments have produced 342 plants, which were transferred to soil. Fifty plants have been regenerated from a single callus line. A smaller effort at plant regeneration from 15 B73-1 protoplast-derived cell cultures produced a total of 9 plants from 2 cell lines. These regeneration numbers for A188 and B73 have been limited more by operator time and culture space than by the regeneration capability of the cell cultures.

Mortality rates have been high after plants were transferred to soil. At this time, only 25 plants (2 of B73, 23 of A188) have grown to maturity, and none of these have been either male or female fertile. Initial greenhouse

conditions were less than optimal for regenerated plants. Although greenhouse conditions have been changed, the cell lines are now getting older. It is not uncommon for the frequency and vigor of regenerated plants to decline with culture age. No mature plants were recovered from the A188 donor culture at the same time that protoplast-derived plants were regenerated. Control plants regenerated from the B73-1 donor culture were not fertile. Karyotype analysis is now in progress to determine if visible cytogenetic changes in the A188 or B73-1 cell lines might be involved.

DISCUSSION

Protoplasts have been isolated from embryogenic maize cultures previously, but subsequent growth and development stopped at either early microcallus stages[14,16] or with the formation of somatic embryos[15]. In this study, plants from protoplast-derived calli have been grown to maturity. The various outcomes achieved in these studies may be due to differences in donor cultures, protoplast isolation procedures, and/or subsequent culture methods. Contrary to results with rice protoplasts[10], morphogenic capacity of protoplast-derived maize calli was identical to that of the donor cultures. Therefore, it seems important to use totipotent cells as sources of protoplasts. Since all 3 cell lines in this study were initiated well before this study began, we do not know if cultures must be a minimum age before their protoplasts will respond to this culture system.

Although undigested cells were present in protoplast preparations, the frequency of callus formation was greater than the frequency of contaminating cells. Undigested cells in protoplast preparations were never observed to divide or even to remain viable longer than 14 days. Recovery of genetically transformed plants using this protoplast system[19] provides unequivocal evidence that callus formation and subsequent plant regeneration originated from protoplasts.

In this study, capability of plant regeneration from protoplast-derived callus was not dependent on genotype. The two unrelated genotypes used, A188 and B73, both responded similarly to these methods for isolation and culture of protoplasts, in contrast to another report[16]. This may have been due to physiological similarities between the A188 and B73 suspension cultures used in this research, which may have obscured genotypic differences in response. Genotypic differences might be apparent if conditions were optimized individually for each genotype. Other studies[20,21] have shown a correlation between genotype and regenerability from callus. A similar type of genotypic correlation also was found in this study: for those cell lines capable of plant regeneration, the A188 cultures produced plants more readily than did the B73 cultures.

Plants were regenerated from 18% of the A188 and 13% of the B73 cell lines recovered from protoplasts. This frequency may have depended upon several factors. Among the variety of cell types in the donor cell cultures which were digested to protoplasts, perhaps only certain cell types were totipotent. Differences among cell lines within each genotype in ability and frequency of plant regeneration may have been due to random sampling during subculture. Another possibility is that the morphogenic capacity of each cell line is determined by some factor in the culture environment.

Problems with fertility of the regenerated plants are more likely due to the donor cell lines and not the protoplast manipulations. Plants regenerated from the donor B73-1 culture were all sterile, so it is not surprising that the 2 B73-1 protoplast-derived plants to reach maturity were also sterile. Many of the regenerated plants from different A188 cell lines shared identical morphological abnormalities (short internodes, asymmetric leaves, empty anthers), which suggests a common origin. Plants can no longer be regenerated from the original A188 donor cell suspension, so a comparison of protoplast-derived plants with callus-derived plants is not possible.

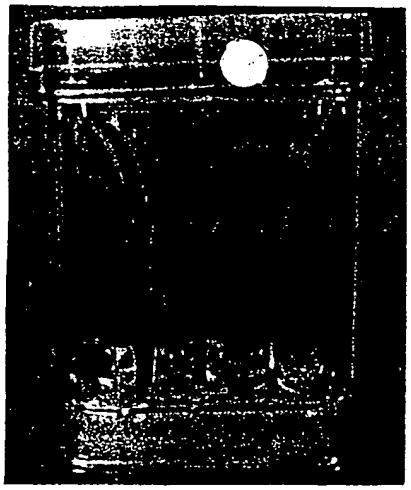

FIGURE 6. Plant regeneration from A188 protoplasts. A188 protoplasts were isolated from embryogenic suspension cultures and grown over BMS feeder layers in N6ap medium. Callus was transferred to MS medium without auxin for 3 weeks. Regenerated plants were grown in plastic boxes, as shown here, on the same MS medium and then transferred to soil.

Modified versions of both NN and MS media have been used previously with maize protoplasts[11–16]. In this study, N6ap resulted in faster callus growth and a higher plating efficiency from protoplasts than either MS or NN. N6ap also promoted formation of somatic embryos, which may have contributed to plant regeneration. Further improvements in plating efficiency or frequency of plant regeneration may be possible with other modifications of N6ap.

The feeder layer system described here greatly increased protoplast plating efficiencies compared to protoplast growth when submersed in liquid or agarose-solidified medium. Support filters over liquid medium permitted good aeration of protoplasts without the stress of dehydration. Feeder cells were still essential in obtaining high protoplast plating efficiency: conditioned medium could not substitute for feeder cells. Protoplasts divided by the third day after digestion and grew rapidly to calli as large as 500 microns in just 14 days.

Protoplast growth was directly correlated with growth of the feeder layer cells. If feeder cells did not grow well, both plating efficiency and growth rate of the protoplasts declined. Feeder cells in liquid medium generally grew better than when embedded in agarose, perhaps due to better aeration and/or faster exchange of nutrients. Protoplasts were separated from nurse cells by only one membrane, which should have allowed maximal exchange of growth-promoting factor(s) from the nurse cells. Pore size of filters can be chosen to permit or exclude exchange of material of known sizes. These feeder layers are easy to assemble rapidly. Media changes for reduction of osmolarity or selection of particular cell clones can be done readily by transferring the support filters underneath the protoplasts to different media.

This protoplast culture system can result in high frequency callus formation from maize protoplasts, and, most importantly, retains the morphogenic capacity of donor cells. In combination with a source of totipotent protoplasts such as the embryogenic suspension cultures used in this study, this enables the recovery of maize plants genetically transformed via direct uptake of DNA or chromosomes, somatic hybridization, or cytoplasmic alterations.

EXPERIMENTAL PROTOCOL

Cell lines. B73 suspension cultures were derived from embryogenic callus cultures initiated from immature embryos approximately 4 years prior to these experiments[22]. They had been maintained in MS medium[23] with 1mM asparagine[24] or in N6ap medium, both with 1 mg/l of 2,4-D. N6ap, derived from Tomes' report[25], consisted of N6 salts[26] plus 6 mM asparagine, 12 mM proline, 1.0 mg/l thiamine-HCl, 0.5 mg/l nicotinic acid, 100 mg/l casein hydrolyzate, 100 mg/l inositol, and 30 g/l sucrose. One cell line (B73-2) consisted of small clumps of rapidly dividing cells (5 to 50 cells per clump) that produced embryo-like structures, which did not develop further into plants. The other cell suspension (B73-1), which had been maintained primarily on solid medium and grown for only 3 months in liquid medium, regenerated plants when grown on medium which promoted plant regeneration (MS with 4% sucrose and without 2,4-D)[20]. The A188 cell line had been initiated as callus from immature embryos 18 months previously, using the procedure of Armstrong and Green[24]. It was maintained on N6ap medium as callus and as suspension culture. Plants were readily produced from cultures grown with either liquid or agar-solidified medium when transferred to regeneration medium.

Preparation of protoplasts. Protoplasts were digested from these suspension cultures, 2 days after subculture, by incubating several grams of cells in mannitol buffer (80 mM $CaCl_2$, 0.5% MES, and sufficient mannitol to bring the osmolarity to 450 milliosmoles) containing 1.0% w/v Cellulase RS, 0.1% Driselase (desalted by column chromatography), 0.1% Pectolyase, and 2 mg/ml bovine serum albumin. Cells were incubated in this solution (1 g cells per 10 ml solution) for 2 to 3 hours with rotary shaking (50 to 90 rpm) at 23°C. Protoplasts were separated from undigested cells by filtering them through either 30 micron nylon mesh or Miracloth. Enzymes were removed by centrifugation (600 rpm in an IEC model 428 centrifuge for 5 minutes) and resuspension of pelleted protoplasts in culture medium. This washing was repeated twice before sampling for yields and plating of protoplasts. Yields of viable protoplasts wefe determined by counting protoplasts stained with fluorescein diacetate[27] in a hemacytometer.

Protoplast culture. Protoplasts were placed on filters (47 mm diameter) over medium with Black Mexican Sweet (BMS) suspension cells[28] as a feeder layer. Except as noted otherwise, 0.25 to 0.4 g of BMS cells were used per plate. Embedded feeders were made by placing 2 ml of medium containing 0.8% low melting temperature agarose (Bio Rad Labs) and BMS cells over 20 ml of solidified medium in 15 × 100 mm petri plates. Liquid spread feeder layers were made by spreading 2 ml of liquid medium containing BMS cells over 20 ml of solidified basal medium. Liquid centered feeders were constructed similarly, but BMS cells were spread only underneath the filters, not over the entire plate surface. Filters were placed directly over feeder layers just prior to adding protoplasts. Identical media (except for agarose) were used for the basal layer, feeder cells and protoplasts within each plate. Media are specified for each experiment. Several types of filters were tested; see Results for their description. Unless specified otherwise, filters were transferred to medium with reduced osmolarity (300 milliosmoles) after 7 days and to medium without added mannitol (180 milliosmoles) after an additional 7 days. Secondary feeders were those plates with BMS cells on 300 milliosmole medium during the second week of protoplast culture. BMS suspensions used as nurse cultures were maintained with twice weekly transfers in MS medium with 2 mg/l 2,4-D. Numbers of calli were counted from 19 to 22 days after protoplasts were isolated. Colonies were gently rinsed off filters into petri plates (100 or 135 mm diameter) and counted with a scanning laser counter (Exotech, Inc. Model 500A). At high rates of protoplast response, laser counts differed from visual counts by less than 10%. In some cases, fresh weight of total callus on each filter was used as an indicator of protoplast response to different treatments.

Plant regeneration. Somatic embryos developed on protoplast-derived callus while still on maintenance medium (N6ap with 1 mg/l 2,4-D). Callus with somatic embryos was transferred to regeneration medium (MS with 4% sucrose and without auxins) and grown under low light levels (10 uE m-2 s-1) for 2 to 4 weeks. Small plants and shoots were selected and transferred to the same medium in clear plastic boxes (Magenta) and grown under bright light (150 μE m-2 s-1) for further development of shoots and roots. When these plants were 5 to 8 cm tall, they were transferred to soil (1 part potting mix (Supersoil):1 part vermiculite) and grown under periodic misting for one week, then raised to maturity in a greenhouse.

Acknowledgments

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Plant Regeneration from Tissue Cultures of Maize[1]

C. E. Green and R. L. Phillips[2]

ABSTRACT

Effective utilization of cell and tissue culture methods in *Zea mays* research requires cultures capable of plant regeneration. These differentiated plants would provide a direct link with conventional genetic and breeding procedures.

Maize callus from embryo scutellar tissues was initiated and maintained on MS medium inorganic components, Straus medium vitamins and amino acids, 20 g sucrose and 8 g agar per liter, and 2 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/liter. Callus has been maintained by subculture every 21 to 28 days and has remained capable of differentiation for 9 months. Regeneration of complete plants was accomplished by subculture of callus to 0.25 mg 2,4-D/liter for 30 days followed by transfer to 2,4-D-free culture medium. At 0.25 mg 2,4-D/liter numerous curled and wrinkled leaves developed. Approximately 200 complete plants have been differentiated after transfer to the 2,4-D-free medium. Root tip cells from five plants indicated that each had 20 chromosomes. After transplantation to soil, 10 to 15% of the plants survived and grew normally. The optimum embryo age for scutellar callus initiation was 18 days post-pollination. Hormone combinations such as 1 mg 2,4-D, 4 mg α-naphthaleneacetic acid (NAA), and 0.05 mg 6-(γ,γ-dimethyl allylamino)-purine (2iP)/liter may increase the efficiency of scutellar callus initiation.

Additional index words: ***Zea mays*** **L., Embryo culture, Scutellum, Hormones, Differentiation.**

THE ability to develop tissue and cell cultures from cereal plants has increased rapidly in recent years. Examples include: somatic callus initiation and plant regeneration in sorghum [*Sorghum bicolor* (L.) Moench] (11), wheat (*Triticum monococcum* L.) (3, 7), rice (*Oryza sativa* L.) (21), and sugarcane (*Saccharum officinarcm* L.) (7); anther culture and haploid plant formation in rice (14), wheat (*Triticum vestivum* L.) (15, 16), and barley (*Hordeum vulgare* L.) (1); and protoplast formation and culture in sugarcane (9).

In maize (*Zea mays* L.), endosperm cultures have been available for many years (20). They have been used for biochemical studies (5, 18, 22) and for protoplast formation and culture (12). More recently, maize somatic callus cultures have been initiated from seedling shoot sections (10), immature inflourescences (6), and mature embryos (6). Plants were not regenerated in these somatic or endosperm callus cultures.

Current cell culture methods have fostered new genetical and biochemical techniques in plants. These techniques coupled with the well-documented genetics and physiology of maize could offer many research opportunities as efficient tissue and cell culture procedures continue to be developed. One of the most critical needs has been to establish callus cultures from which complete plants could be regenerated. This would provide a direct link between cell culture research and conventional genetic and breeding procedures. This paper describes parameters for initiating and maintaining callus cultures from immature maize embryo scutellum and the subsequent regeneration of complete plants.

MATERIALS AND METHODS

Primary genotypes used in this study included the field-corn inbred A118 (*r—r*; colorless aleurone, embryo scutellum and plumule, and dilute sun red plant color) and the homozygous R-navajo (*R—nj*; pigmented aleurone crown, embryo scutellum and plumule, and seedling) stocks. Field-corn inbreds A619, A632, B9A, and W64A were obtained from J. Geadelmann, Dep. of Agron. and Plant Genet., U. of Minn.

Ears were removed from field or greenhouse-grown plants at 14 through 24 days after pollination. Within 30 minutes after removal from the plant, they were broken into 5 to 8 cm segments. They were then sterilized for 20 min by submerging in a solution of 1 g Al lab detergent (A & L Laboratories, Minneapolis, Minn.)/100 ml 2.5% sodium hypochlorite. The ear segments were then transferred through three sterile deionized-distilled water rinses. The isolation of embryos was usually begun immediately; however, sterile ears could be stored without apparent damage to the embryos for at least 12 hours at 4 C.

Immature embryos were isolated from the ear by cutting off the kernel crown, removing the endosperm with a narrow spatula, and then transferring the embryo, located at the kernel base, onto culture medium. Embryos were placed on the solid culture medium with the rounded scutellar side exposed and the flat plumule-radicle axis side in contact with the medium.

Callus was initiated and maintained on MS culture medium which contained the inorganic components of Murashige and Skoog medium (13); 7.7 mg glycine/liter, 1.98 g L-asparagine/liter, 1.3 mg niacin/liter, 0.25 mg thiamine-HCl/liter, 0.25 mg pyridoxine-HCl/liter, and 0.25 mg Ca pantothenate/liter from Straus medium (4, 19); 20 g sucrose/liter; and 8 g agar/liter. Varying concentrations of the plant hormones, 2,4-dichlorophenoxyacetic acid (2,4-D), p-chlorophenoxyacetic acid (p-CPA), α-naphthaleneacetic acid (NAA), and 6-(γ,γ-dimethylallylamino)-purine (2iP) were used. The medium was adjusted to pH 6.0 with 0.4 *N* NaOH and autoclaved for 20 min at 15 psi.

The embryos and subsequent callus cultures were incubated at 28 to 30 C with a 16/8 hour photoperiod from cool-white fluorescent lights with an intensity of 2,000 lux. Callus cultures were maintained by subculturing every 21 to 28 days to MS containing 2 mg 2,4-D/liter.

Root tips from differentiated plants were prefixed at 4 C in saturated 8-hydroxyquinoline for 4 hours, transferred to fresh Farmer's solution (3 parts 95% ethanol:1 part glacial acetic acid), fixed for 24 hours at room temperature, and stored at —10 C in 70% ethanol. Chromosome counts were made on metaphase cells from squashed root tips stained with propionocarmine and observed by phase-contrast microscopy.

RESULTS AND DISCUSSION

Culture Initiation

Initial success in establishing totipotent callus cultures from maize was achieved by aseptically isolating 20-day post-pollination embryos from the cross A118 × *R-njR-nj*. With the plumule-radical axis side placed in contact with MS medium containing 1,

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2, 4, or 6 mg 2,4-D liter, embryos were incubated at 28 to 30 C. This orientation allowed germination, but suppressed subsequent embryo growth and promoted callus formation from the scutellum. In comparison, when the rounded, scutellar embryo surface was placed in contact with the medium and the plumule-radical axis was oriented upwards, rapid germination and extensive shoot and root growth occurred, even in the presence of 4 to 6 mg 2,4-D/liter. No scutellar callus was formed, although slow growing compact yellow callus frequently developed from roots and/or the first node of the shoot after 4 weeks incubation. These calli were generally similar to the somatic cultures previously reported (6), which were easily maintained and differentiated numerous roots, but not shoots.

Culture initiation began with enlargement of the entire exposed scutellar surface, which resulted in a 6 to 8 mm diameter dome-shaped scutellum within a few days. After 10 days, the scutellum surface had developed a bumpy and irregular shape and by 14 days, nodular white or pale-yellow callus was visible in localized regions. These primary cultures developed 1 to 2 cm callus masses after 30 days, which frequently exhibited early organizational events; such as localized chlorophyll development and the formation of occassional light-green 1 to 2 mm long leaves from the chlorophyll regions.

Table 1. Ability of immature embryos from two maize lines to form scutellar callus cultures capable of plant regeneration.

Genotype	Source	Embryos isolated	Callus clones at 2	Clones differentiating leaves at 0.5
			— mg 2,4-D/liter —	
A188 × R-njR-nj	Field, 1973	7*	5	3 (3)‡
	Field, 1974	220†	174	77 (8)
A188	Greenhouse, 1974	106†	91	29 (15)
	Field, 1974	260†	141	55 (5)

* 20 day post-pollination embryos. † 18 day post-pollination embryos. ‡ ( ) represents the number of totipotent callus clones tested on MS medium with 0 mg 2,4-D/liter for plant differentiation. All tested clones differentiated complete plants

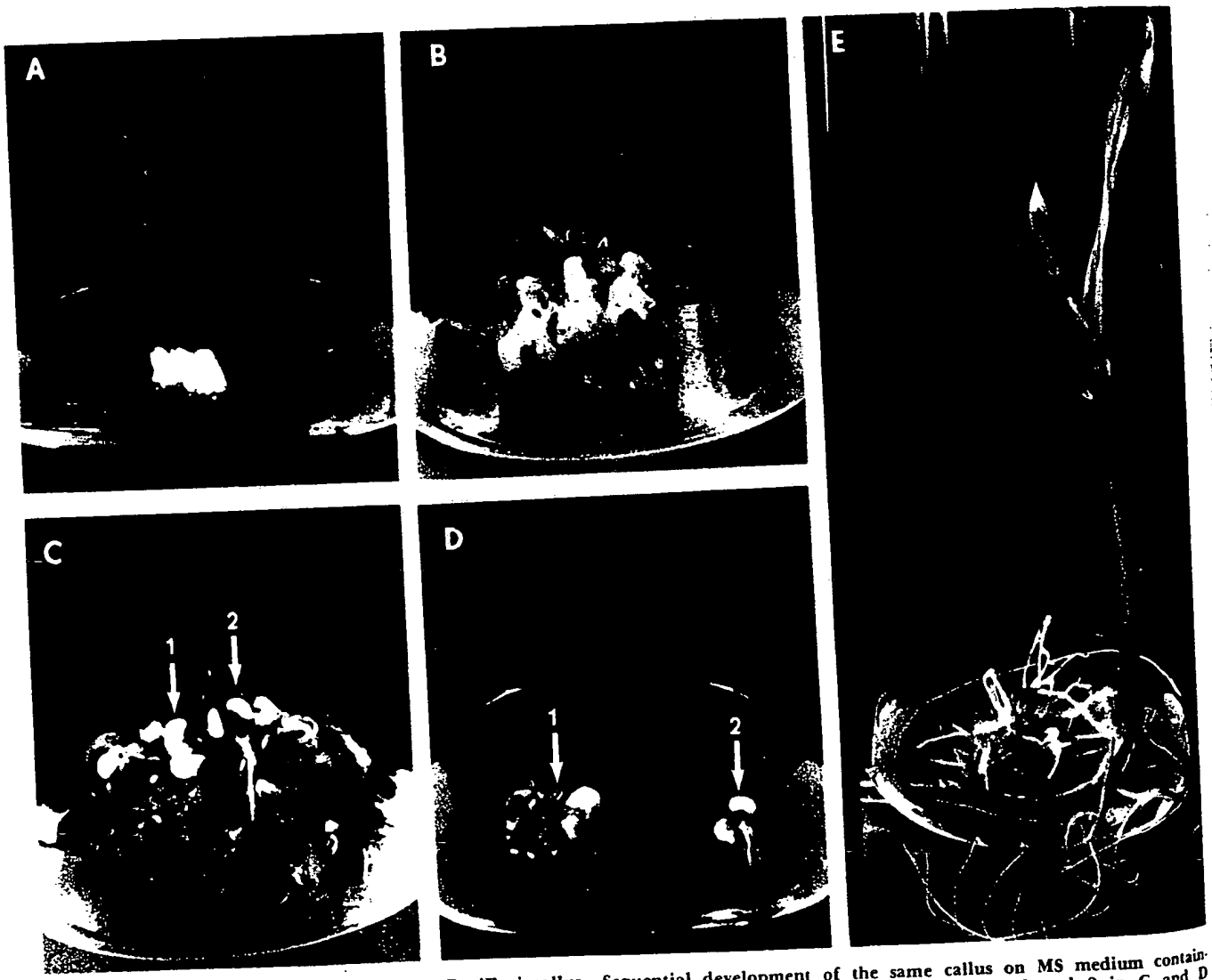

Fig. 1. Plant differentiation from A188 × *R-njR-nj* callus. Sequential development of the same callus on MS medium containing 0.25 mg 2,4-D/liter after 0, 20, and 30 days incubation, is shown in A to C, respectively. Arrows 1 and 2 in C and D designate the tissues subcultured to 2,4-D-free medium in D. E shows the differentiation in D after 20 days.

Totipotent callus cultures were most successfully initiated from the scutellum on MS medium containing 2 mg 2,4-D/liter. Medium containing 4 mg 2,4-D/liter was less effective. MS containing 1 mg 2,4-D/liter allowed increased embryo shoot and root growth and limited callus formation from the scutellum. Embryo shoot and root growth was minimal and the scutellum produced slow growing callus on medium containing 6 mg 2,4-D/liter. No plants were differentiated from these cultures. During subculture, callus was easily separated from scutellar tissue, which had not grown, and the remainder of the embryo. The original A188 × *R-njR-nj* cultures have been maintained for 19 months on MS medium with 2 mg 2,4-D/liter by subculturing every 21 to 28 days. Localized areas of chlorophyll and small leaves have continued to form, especially near the end of each culture period. This organization was apparently associated with the gradual depletion of 2,4-D in the culture medium.

Totipotent scutellar callus has been initiated from 8 independent plant populations. These were A188 × *R-njR-nj* crosses and sibbed or selfed A188 (Table 1). Among the populations, callus initiation on MS medium containing 2 mg 2,4-D/liter ranged from 54 to 85% of the embryos tested. These callus clones were maintained by subculture and their totipotency tested by subsequent subculture to MS medium containing 0.25 mg 2,4-D/liter. After 30 days incubation, the clones differentiating leaves varied from 21 to 43% of the isolated embryos.

The plumule and radicle tissues of embryos had a characteristic fate during scutellar callus initiation which was related to embryo age and development at isolation. The shoot and root primordia in A188 × *R-njR-nj* or A188 embryos isolated from ears after 20 days development formed 2 to 3 cm plumules and 0.5 cm radicles during the initial 10 days of incubation on MS medium containing 2 mg 2,4-D/liter. These tissues did not elongate further during subsequent incubation and were usually necrotic after 30 days. No plumule or radicle elongation occurred during incubation of 14-day embryos while 5 to 8 cm plumules and 0.5 cm radicles developed during incubation of 24-day embryos.

Differentiation

The differentiation and growth of plants from A188 × *R-njR-nj* scutellar callus is illustrated in Fig. 1. The visibly unorganized 0.5 cm diameter callus in Fig. 1-A was obtained from MS medium containing 2 mg 2,4-D/liter and transferred to MS containing 0.25 mg 2,4-D/liter. By 10 days, the rapid growth of this callus was accompanied by localized chlorophyll development and the formation of small, 1 to 2 mm leaves. The growth and development at 20 days (Fig. 1-B) included many light-green leaves and white compact structures that resembled the organized scutellum of the original embryos. By 30 days, many curled and wrinkled leaves, additional white scutellar-like structures (Fig. 1-C, arrows 1 and 2) and short roots had formed.

The differentiation of complete seedlings was accomplished by transferring cultures with many small leaf structures from MS containing 0.25 mg 2,4-D/liter to 2,4-D-free medium (Fig. 2-B). In order to closely observe seedling development, the organized culture in Fig. 1-C was subdivided into 12 small fragments which were inoculated onto 2,4-D-free MS medium. Two of these (Fig. 1-D, arrows 1 and 2) cor-

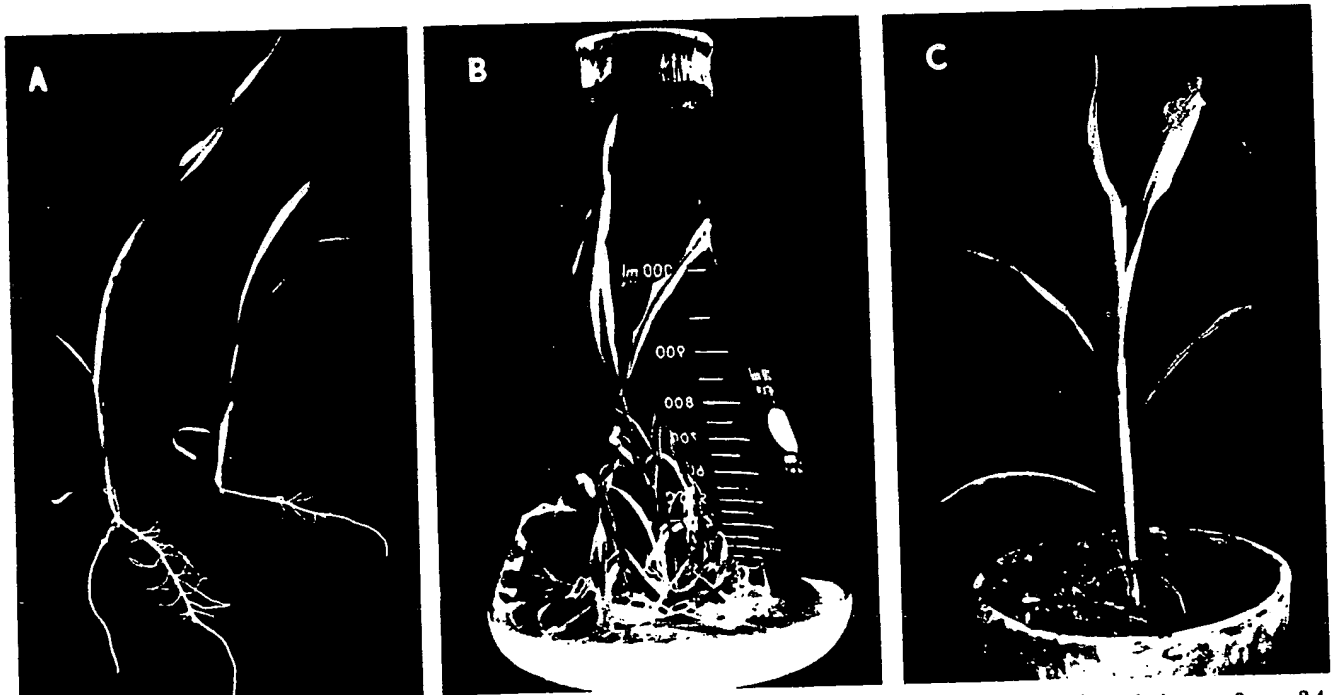

Fig. 2. **Differentiated A188 × *R-njR-nj* plants. A typical root-shoot development; B, multiple plant differentiation at 0 mg 2,4-D/liter; C, five-leaf-stage differentiated plant established in soil.**

Table 2. Influence of embryo age and genotype on scutellar callus formation.

Inbred	Post-pollination embryo age, days					
	14	16	18	20	22	24
	— embryos forming differentiating cultures*, % —					
A188	18	15	21	14	9	0
A619	0	5	6	5	0	0
A632	0	0	0	0	0	0
B9A†	0	5	8	0	0	0
W64A	0	0	11	0	0	0

* Each value represents the percent of 60 embryos. † Subsequently, these B9A cultures died during the fourth month of growth (fourth subculture).

respond to the tissues indicated by the arrows in Fig. 1-C. The plant shown in Fig. 1-E was one of seven which differentiated during 20 days incubation. This plant emerged between the lobes of the white scutellar-like tissue (Fig. 1-D, arrow 2) and had attached roots. Typical shoot-root associations and development in the differentiated plants is shown in Fig. 2-A. To date, approximately 200 seedlings have been differentiated from A188 × *R-njR-nj* callus, and the original cultures remain totipotent after 19 months. Cultures, which did not form plants on 2,4-D-free medium, frequently developed extensive roots as shown by the tissues on the left in Fig. 1-E. Recently, these results have been independently confirmed by the initiation of totipotent cultures and subsequent plant differentiation from the maize line Alhexo Single Kernel Cycle IV in another laboratory (2). In addition, primary cultures obtained from inbred W64A scutellum also have been observed to regenerate maize plants. These cultures, however, were not successfully maintained by subculture (23).

When two-leaf-stage differentiated A188 × *R-njR-nj* or A188 plants were transplanted to steam sterilized soil 10 to 15% of the plants became established and grew (Fig. 2-C). This limited success was apparently due to inadequate root development in the culture medium. Roots were frequently thin and total root mass was small in relation to shoot tissues. Differentiated plants grown in soil had wide leaves, ears, and tassels. Pollen examined from one plant was 90 to 95% fertile. Although many abnormal leaf shapes were observed during plant differentiation, no albino, straited, or chimeral leaves or plants were observed. Chromosome counts in root tip cells from five differentiated plants indicated that each had 20 chromosomes. Meiotic tissue has not yet been examined cytologically nor have crosses been attempted to recover progeny from differentiated plants.

Preliminary results indicated that p-CPA at 2 and 4 mg/liter was less effective than 2,4-D for scutellar callus initiation from 18-day A188 × *R-njR-nj* embryos. Although callus formation with p-CPA was less prolific, it became more highly organized with numerous small leaves present after 30 days. Plant differtiation from these cultivars was vigorous on MS medium with 0 mg p-CPA/liter. When NAA and 2iP were combined at 2, 4, or 8 mg/liter and 0.05 mg/liter respectively, scutellar callus initiation was very limited. Promising results were obtained, however, from the hormone combination 1 mg 2,4-D/liter, 4 mg NAA/liter, and 0.05 mg 2iP/liter wherein 25 of 32 embryos developed callus which has been maintained through three subcultures to date Leaves developed rapidly in 18 of these callus clones when subcultured to MS containing 1 mg NAA/liter and 0.05 mg 2iP/liter. These preliminary results suggest that differentiating cultures can be obtained with hormones other than 2,4-D and that hormone combinations may increase the efficiency of culture initiation.

Influence of Embryo Age and Genotype

The influence of embryo age on the formation of callus and plant differentiation was studied by isolating embryos from the field grown inbreds; A188, A619, A632, B9A, and W64A (Table 2), which had been sib or self-pollinated. Embryos were isolated from ears harvested at 2-day intervals from 14 through 24 days after pollination. Although embryo size varied slightly between genotypes, typical embryo lengths were as follows: 1 mm at 14 days, 3 mm at 18 days, and 5 mm at 22 days. Sixty embryos from each genotypes at each age were explanted onto MS medium with 2 mg 2,4-D/liter and incubated for 30 days. Scutellar calli were subcultured to MS medium with 2 mg 2,4-D liter for 21 days and then to medium with 0.25 mg 2,4-D/liter to detect cultures capable of plant differentiation. Variability existed between genotypes in ability to initiate differentiating cultures. For example, A188 formed differentiating cultures from embryos at all ages except 24 days, while none were recovered from A632. However, the optimal embryo age for totipotent culture initiation was 18 days for A188, A619, B9A, and W64A. Maize embryos are approaching maturity in embryological development and size at 22 days after fertilization. The poor response of older embryos may reflect a requirement for rapidly growing scutellar tissue for culture initiation.

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Heritability of Nitrate Reductase and Cyanide Levels in Seedlings of Grain Sorghum Cultivars[1]

H. V. Eck, E. C. Gilmore, D. B. Ferguson, and G. C. Wilson[2]

ABSTRACT

Seedlings of 41 cultivars of grain sorghum [*Sorghum bicolor* (L.) Moench] including 30 hybrids and all but two of their parents were grown in a growth chamber; harvested 10, 14, and 18 days after planting; and assayed for hydrolizable cyanide and *in vitro* and *in vivo* nitrate reductase activity (NRA). The genotype × age variance component was essentially equal to the genetic variance for each character, which suggest that genotype × environment interactions are major components of the phenotypic variance. Cyanide level was highest in 10-day-old plants and dropped sharply in 14-day-old plants. Broad-sense heritability of hydrolizable cyanide was high (0.89 to 0.95) for all three ages of plants. Narrow-sense heritability indicated the cyanide content of 10-day-old plants could be changed 0.76 units for each unit change in the midparental value, but expected progress decreased markedly for 18-day-old plants. Broad-sense heritability of *in vitro* NRA was high at all ages, and narrow-sense heritability was moderate to low. Thus, significant genetic advance could be expected from selection among pure lines and hybrids, but nonadditive genetic variance would reduce progress in hybrids based on selection of their parents to a moderate level. *In vivo* NRA was only 20% of *in vitro* activity. Heritabilities, in general, also were lower for *in vivo* than for *in vitro* NRA.

Additional key words: **Selection, Yield, Enzymes, Plant breeding, Hybrids.**

ENZYME activities may have potential as selection criteria in developing cultivars or hybrids of crop plants (5, 7). Hageman et al. (7) have discussed the bases for breeding for enzyme activity. The enzyme nitrate reductase (NR) is one of the more promising enzymes because reduction of nitrate to nitrite (catalyzed by NR) has been shown to be the limiting step in N assimilation in plants (1). Nitrate reductase activity (NRA) has been measured in numerous plant species (10) and has been related to parameters such as total reduced N in vegetation and grain in wheat, *Triticum aestivum* L., (3); grain yield and grain protein in corn, *Zea mays* L., (4); and total dry weight accumulation in ryegrass, *Lolium perenne* L., (2).

Warner (13) found that in corn, NRA is highly heritable and subject to genetic manipulation. Warner (13) and Eilrich and Hageman (6) determined that the genetic potential for NR could be assessed in the seedling stage. The assay of NRA is fast and relatively inexpensive as compared with yield testing. Success in screening genotypes for yield on the basis of their NRA would depend on the existence of genetic variance for NRA in a population and the covariance of NRA and yield.

Cyanogenic glycosides (dhurrin) in leaf tissue of grain sorghum [*Sorghum bicolor* (L.) Moench] and sudangrass [*Sorghum sudanense* (Piper) Stapf] have made it difficult to obtain valid or reproducible measurements of NRA in such species. Cyanide released upon homogenization of the tissue inhibits NR. In 1970, however, Maranville (12) reported that adding Ni to the extraction medium would complex the cyanide and thus permit NRA in sorghum to be measured. Eck and Hageman (5) modified Maranville's procedure and found that they could assay tissue containing as much as 7.2 μmol of free cyanide (approximately 35 μmol of total hydrolizable cyanide) per gram fresh weight without results being affected by endogenous cyanide. They also investigated Klepper's (10) *in vivo* method for measuring NRA, but found it more variable than the *in vitro* method.

Sorghums used as livestock forage have been investigated for hydrolizable cyanide (hydrocyanic acid potential), but little information is available for grain sorghums. Loyd and Gray (11), working with two sudangrass cultivars and a sudangrass × sorghum, reported that cyanide content was highest in 1-week-old plants and then decreased until maturity. Eck and Hageman (5), studying 17, 10-day-old sudangrass cultivars, found hydrolizable cyanide levels ranging from 2.6 to 16.4 μmol/g fresh wt.

The objective of this study was to determine the genetic variances and heritability for *in vivo* and *in vitro* NRA and total hydrolizable cyanide in a population of grain sorghum genotypes.

Contribution from the soil, water, and air sci., Southern Region, ARS, USDA, in cooperation with the Tex. Agric. Exp. Sta., Tex. A&M U. Received Nov. 4, 1974.

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SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM THE FRIABLE CALLUS OF ZEA MAYS

Corn R2

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One of the interesting developments in recent years in cereal tissue culture has been the occurrence of somatic embryogenesis (1,2). Although relatively new in cultures of cereals and grasses, this phenomenon was described in detail by Steward and his co-workers (3) and other investigators approximately twenty years ago. Methods which reliably facilitate somatic embryogenesis from friable callus and suspension or protoplast cultures of cereal crops would significantly enhance the prospects for genetic engineering using molecular technologies on these species.

Tissue culture methods have improved in corn so that now it is possible to regularly generate and maintain friable callus cultures (4). These new cultures have thus far been developed from the inbred A188 and are distinguished from previous organogenic as well as friable cultures of corn by their capacity to regenerate plants by somatic embryogenesis. These cultures appear to be very undifferentiated in that they are highly friable and little organization is visible to the eye. Examination by light and scanning electron microscopy, however, reveals somatic embryos which follow very closely the developmental sequence established for the zygotic embryos of corn (5).

These friable calluses develop directly from the surface of the scutellum of immature embryos or as spontaneous sectors growing from established organogenic tissue cultures of the type reported earlier (6,7). They have been generated and maintained on both MS and N6 mediam containing 2,4-D. These cultures may not exhibit obvious embryogenic activity initially but usually within two weeks of their generation microscopic examination reveals a variety of globular structures on their surface. Established cultures have a rapid growth rate which requires that they be subcultured on a fresh medium every two weeks.

The established cultures exhibit both a high degree of embryogenic activity as well as the preservation of embryogenic potential for long periods. The initial cultures have continued to form large numbers of embryos for over two years. A callus ready for subculture on a fresh medium may have hundreds of embryos at various stages of development on its surface. The earliest visible stage are approximately 100μ in diameter (Figure 1A). These may occur more or less randomly on the surface of the callus or be clustered together at high density. The continued development of these globular structures into embryos is evidenced by the differentiation of the suspensor, scutellum, and embryonic axis (Figure 1B). The suspensor always provides the direct association between the embryo and the callus.

Embryonic development up to the coleoptilar stage (8) progresses quite readily on either MS or N6 media containing 2% sucrose and 0.5 to 1.0 mg/l 2,4-D. Development beyond this stage is very anbormal unless the callus is transferred to a N6 medium lacking 2,4-D but containing 6% sucrose, to increase the osmolarity of the medium. Maturation occurs rapidly on the elevated sucrose and after 10-14 days the largest embryos have developed to sizes similar to those found in seeds. Clearly recognizable features of these embryos include a well-developed scutellum and embryonic axis with a very prominent coleoptile (Figure 1C). Embryo germination is severely retarded on a medium with a high sucrose composition but when transferred to MS or N6, lacking hormones and

with 2% sucrose the mature embryos germinate rapidly. Shoot and root growth usually occur simultaneously and the young plants are grown in a 1:1 mixture of sterile soil and vermiculite (Figure 1D). Among about a thousand plants regenerated from these friable embryogenic cultures, approximately 150 have been grown to maturity, evaluated for abnormalities, and crossed to produce progeny for further genetic analysis.

Suspension cultures have also been generated from the friable embryogenic callus. They have been established and maintained in the MS medium containing 1mg/l 2,4-D and 2% sucrose. These suspensions are well-dispersed and composed of cell aggregates ranging from 2mm in diameter to single cells. Most of the single cells are quite elongated and do not appear to undergo further growth. The cell aggregates are usually composed of small oval cytoplasmically rich cells. Embryo development, and then only to the early globular stage, was observed very infrequently in the suspension cultures. Embryogenesis was vigorous, however, in callus cultures grown from suspension cells plated on agar-solidified MS medium. Plants were regenerated from the resulting somatic embryos.

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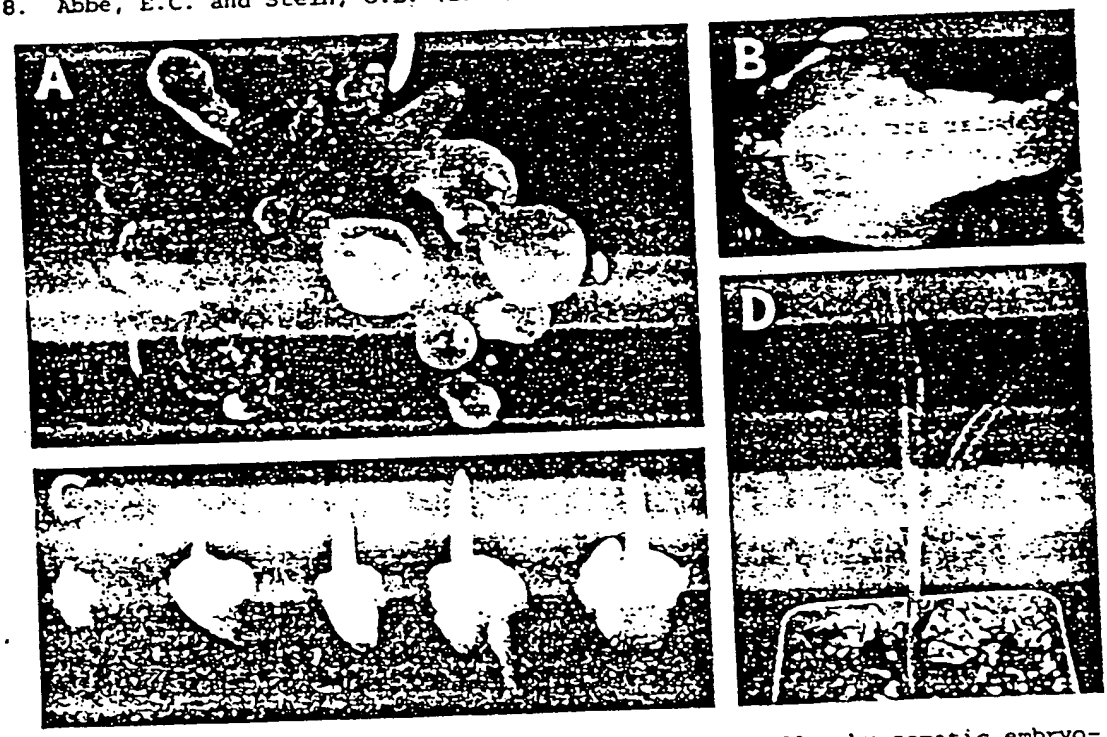

FIGURE 1. Regeneration of corn plants from friable callus by somatic embryogenesis. (A) Friable callus and globular embryos, 40X (B) Coleoptile stage embryo 40X (C) Embryo maturation at 6% sucrose, 1X (D) Plant regeneration from somatic embryo.

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Formation of callus and somatic embryos from protoplasts of a commercial hybrid of maize (*Zea mays* L.)

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Summary. Protoplasts isolated from a totipotent embryogenic cell suspension culture of *Zea mays* L. (cultivar 'Dekalb XL82') underwent sustained cell divisions when cultured in liquid as well as agarose media. Optimal colony formation (5%) occurred in a liquid medium containing 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). A soft and unorganized callus was formed when the protocolonies were transferred to agar solidified suspension maintenance medium. Compact, organized and yellow to pale green folded structures and somatic embryos were formed upon subsequent transfer of this callus to a low 2,4-D medium. Clusters of somatic embryos germinated precociously but no plants were recovered.

Key words: Cereals – Gramineae – Maize – Protoplasts – Somatic embryogenesis – Suspension culture – *Zea mays*

Introduction

Sustained cell divisions and callus formation have been reported previously in protoplasts isolated from suspension cultures of *Zea mays* (Chourey and Zurawski 1981; Ludwig et al. 1985). So far this has been possible only in a single cultivar of maize, namely 'Black Mexican Sweet'.

Recently, transient expression of foreign genes in, as well as stable transformation of, protoplasts isolated from a 'Black Mexican Sweet' suspension culture of maize has been achieved by electroporation (Fromm et al. 1985, 1986). The cell lines used in each of the above experiments were comprised predominantly of aneuploid and polyploid cells, and showed no competence for morphogenesis or plant regeneration. The use of embryogenic cell suspension cultures has previously been shown to be advantageous for the recovery of somatic embryos and plants from protoplasts of the Gramineae (Vasil and Vasil 1980; Srinivasan and Vasil 1986; IK Vasil and Vasil 1986; Yamada et al. 1986).

In order to further exploit recent successes in the somatic hybridization (Ozias-Akins et al. 1986; Tabaeizadeh et al. 1986) as well as transformation of gramineous species (Fromm et al. 1986), it is important to extend these advances to protoplasts isolated from morphogenically competent cell lines of maize. In a previous report we described the regeneration of plants from an embryogenic cell suspension culture of a commercial hybrid cultivar ('Dekalb XL82') of maize (V Vasil and Vasil 1986). We now report sustained cell divisions in protoplasts, and the formation and precocious germination of somatic embryos in protoplast-derived calli of this morphogenic cell line of maize.

Materials and methods

Isolation and maintenance of cell suspension cultures

An embryogenic cell suspension culture, designated as line M1, was established from Type II friable embryogenic callus obtained from immature embryos of *Zea mays* L. in September-October, 1984 (V Vasil and Vasil 1986). Two separate cell lines, designated as M2 and M4, were isolated from line M1 within nine months after its initial establishment. The suspension cultures were maintained in N_6 medium (Chu et al. 1976) that was supplemented with 1.25 mg/l 2,4-D, 575 mg/l (5 mM) L-proline, 200 mg/l casein hydrolysate, 2% sucrose and 0.02 mg/l abscisic acid (ABA) (= suspension maintenance medium). For weekly subcultures, 8 ml of the suspension was pipetted into 25 ml fresh suspension maintenance medium in a 125 ml Erlenmeyer flask. The cultures were incubated in the dark at 27 °C on a gyratory shaker at 150 rpm. Fresh weights of the suspensions were determined by pipetting 1 ml samples on to pre-weighed filter papers, draining under vacuum, and weighing again.

Table 1. Growth, yield of protoplasts and spontaneous fusion, and efficiency of colony formation from protoplasts in lines M1, M2 and M4 (data based on two separate experiments, each with three replicates)

Suspension line (age in months)	Fresh weight (mg/l) after 7 days	Protoplast yield/g fresh wt	Spontaneous fusion (%)	Colony formation (%)
M1 (15)	572	2×10^6	5–10	3–5
M2 (6)	688	3×10^6	10–15	3
M4 (6)	812	5×10^6	20–30	4

Protoplast isolation and culture

The suspension cultures were subcultured 2–3 days prior to protoplast isolation. Four to 5 ml of the settled cells (1 gm fresh weight) were mixed with 50 ml filter-sterilized enzyme solution and distributed equally in three 100×15 mm Petri dishes. Protoplasts were released after 5–6 h at room temperature on a gyratory shaker at 50–60 rpm. The enzyme solution was prepared by dissolving cellulase Onozuka RS (3%) and pectinase Serva (1%) in a buffer solution consisting of 7 mM $CaCl_2 \cdot 2\,H_2O$, 0.7 mM $NaH_2PO_4 \cdot H_2O$, 0.5 M mannitol and 3 mM MES at pH 5.6. The $CaCl_2 \cdot 2\,H_2O$ tended to cause crystal formation and since its presence was not found to be critical, it was eliminated from the solution in later experiments.

The protoplast-enzyme mixture was filtered first through a double layer of Miracloth and then successively through 100, 50 and 25 µm stainless steel filters to remove undigested cells and larger spontaneous fusion products. The protoplasts were collected by centrifugation (100×g, 3 min) and washed three times with the buffer solution. The washed protoplasts were counted in a Batch Counting Chamber (Hausser Scientific, PA). Calcofluor White ST (0.1%) was used to determine the presence or absence of cell wall in the protoplasts (Nagata and Takebe 1970). Kao and Michayluk's (1975) modified KM medium (Vasil and Vasil 1980), adjusted to pH 5.6 and filter sterilised, was used for the culture of protoplasts. The KM medium was supplemented with 0.45 M glucose, and 2,4-D (0.1–2 mg/l) alone or in combination with zeatin (0.25 mg/l). The osmolarity of the enzyme solution, buffer, and the culture medium was adjusted to 650 m osm · kg/l H_2O. The protoplasts were cultured at a density of $1–3\times10^5$/ml in 1–1.5 ml liquid medium or in different concentrations (0.3 to 1.2%) of low-melting-point Seaplaque agarose (FMC Corp., Rockland, ME) in CoStar dishes (60×15 mm). The cultures were sealed with Parafilm and incubated in the dark at 27 °C in growth chambers.

Fresh medium (0.5 ml) with 0.3 M glucose was added to the cultures after 10–18 d. After another week, the protoplast-derived colonies were pipetted (0.75–1 ml) on agar (0.8%) solidified suspension maintenance medium with 2,4-D (0–1.25 mg/l), either alone or in combination with zeatin or benzylaminopurine (0.1–1 mg/l). For growth and organization, small pieces (ca. 5 mm^3) of the resulting 3–4 week old calli were transferred to various media.

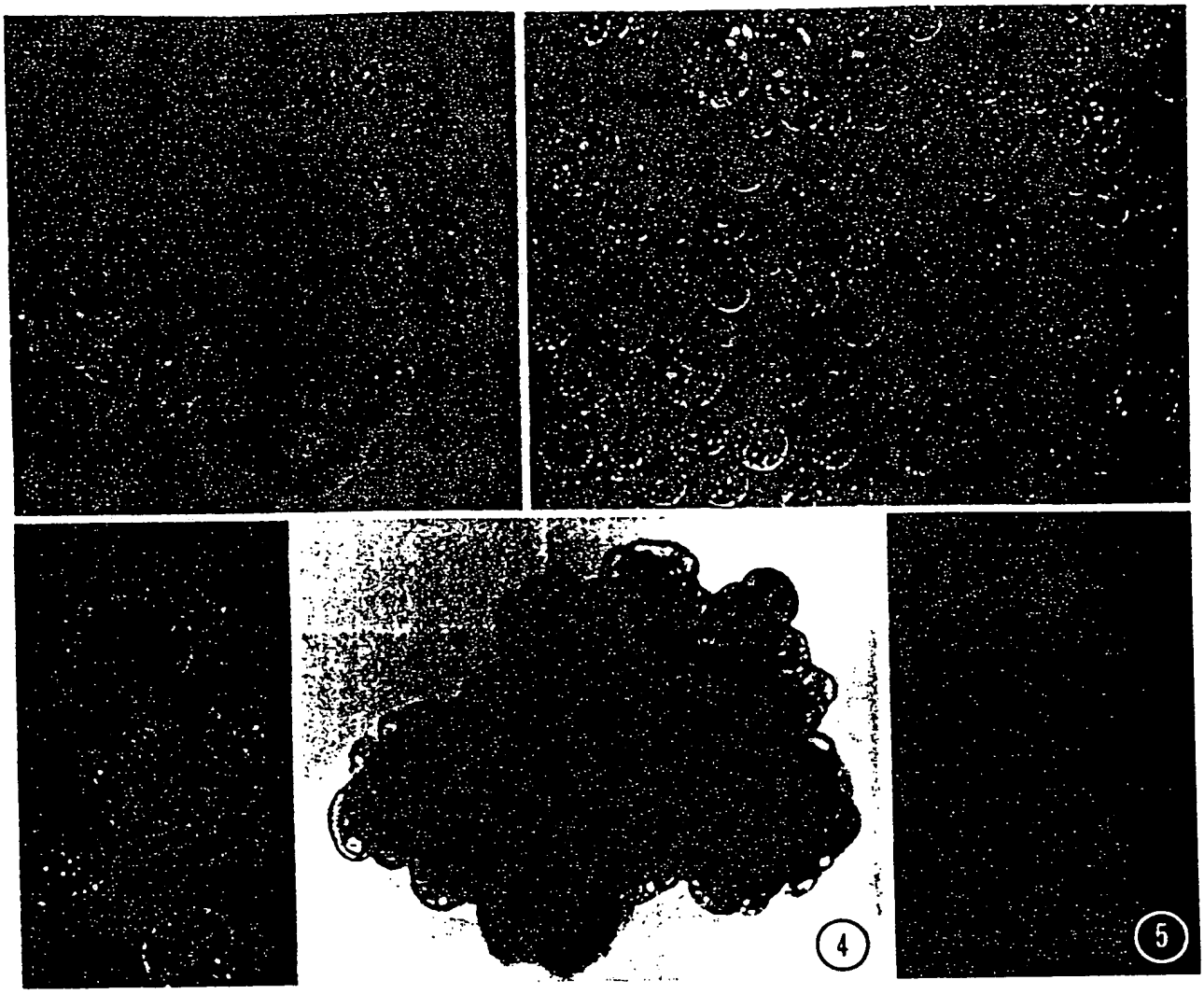
Results

Formation of protocolonies

The original suspension culture line M1 was embryogenic and, as previously described (V Vasil and Vasil 1986), produced somatic embryos and plants upon plating on agar solidified medium. The suspension culture maintained its capacity to produce somatic embryos 15 months after isolation. The suspension was comprised of small groups of thin-walled, highly cytoplasmic and starch-containing embryogenic cells (Fig. 1) and some (10–15%) elongated, thick-walled cells. The thick-walled cells neither divided nor formed protoplasts during enzymatic digestion. Almost all, the thick-walled cells were removed effectively during washing and sieving and were seen only rarely in washed protoplast preparations. The freshly isolated protoplasts (Fig. 2) were devoid of any remaining cell wall material as demonstrated by staining with Calcofluor White. Protoplast yields ranged from 4×10^5 to 6×10^6 protoplasts/gm fresh weight of the suspension culture. Protoplast yield and the degree of spontaneous fusion varied in the different experiments even though identical procedures were used each time both for the maintenance of the suspension culture as well as for the isolation of protoplasts.

The protoplasts were 15–35 µm in diameter, highly cytoplasmic and contained many small starch grains (Fig. 2). A majority of the protoplasts formed cell walls within two days and became oval in shape. Two-celled structures were first seen around the fifth day after culture (Fig. 3). Further divisions then followed in quick succession. The resulting protocolonies resembled the embryogenic cell groups present in the suspension cultures (Fig. 4). Frequently, single file chains of 4–8 cells also were formed. A large number of protocolonies were produced within the first two weeks of culture.

Protoplasts cultured in liquid media tended to aggregate at the periphery of the culture dish which made it difficult to accurately determine plating efficiencies. Therefore, the total number of apparently independent protocolonies that were produced during the first two weeks of culture were counted. The efficiency of colony formation was expressed as the percentage of total plated protoplasts which formed colonies. In basal KM medium the protoplasts remained round and neither regenerated a cell wall nor divided. When 0.1 mg/l 2,4-D was added to the medium, a majority of the protoplasts formed cell walls, became oval in shape and occasionally divided (0.01% colony formation) to form 2- to 8-celled groups which failed to develop further. The



Figs. 1–5. Formation of colonies from protoplasts isolated from embryogenic cell suspension cultures of *Zea mays* L. 1 Groups of highly cytoplasmic embryogenic cells from the suspension culture (×510). 2 Freshly isolated protoplasts. Note absence of undigested cells (×410). 3 A 2-celled group following the first division of the protoplasts (cultured in medium with 0.6% agarose) (×465). 4 Protocolony (×130). 5 An organized, globular somatic embryo (×610)

highest frequency of colony formation (4–5%) was obtained with 0.5 mg/l 2,4-D. Higher concentrations of 2,4-D (1–2 mg/l) supported initial divisions in protoplasts (0.1–3% colony formation) but were inhibitory for continued cell divisions and growth. Zeatin (0.2 mg/l) did not generally prove useful, and it caused browning of the protocolonies when used with 0.5 mg/l 2,4-D.

The frequency of colony formation was not improved by culture of protoplasts in media gelled with various concentrations of agarose. Optimum colony formation (3.5–4%) was obtained with 0.3% agarose; concentrations above 0.3% inhibited both the initial divisions as well as further growth of the protocolonies.

The three suspension culture lines (M1, M2 and M4) showed differences in growth as measured by fresh weight increase, the yield of protoplasts, and the degree of apparent spontaneous fusion during protoplast isolation (Table 1). Protoplast yields from lines M2 and M4, which showed higher fresh weight values, were also higher than those from M1. Although protoplast yields were considerably higher in M4, this line also showed a higher frequency of spontaneous fusions. The three cell lines did not differ significantly in the frequency of colony formation.

Sustained divisions in cultured protoplasts were obtained in each of the 14 separate isolations, with a maximum plating efficiency of 5%.

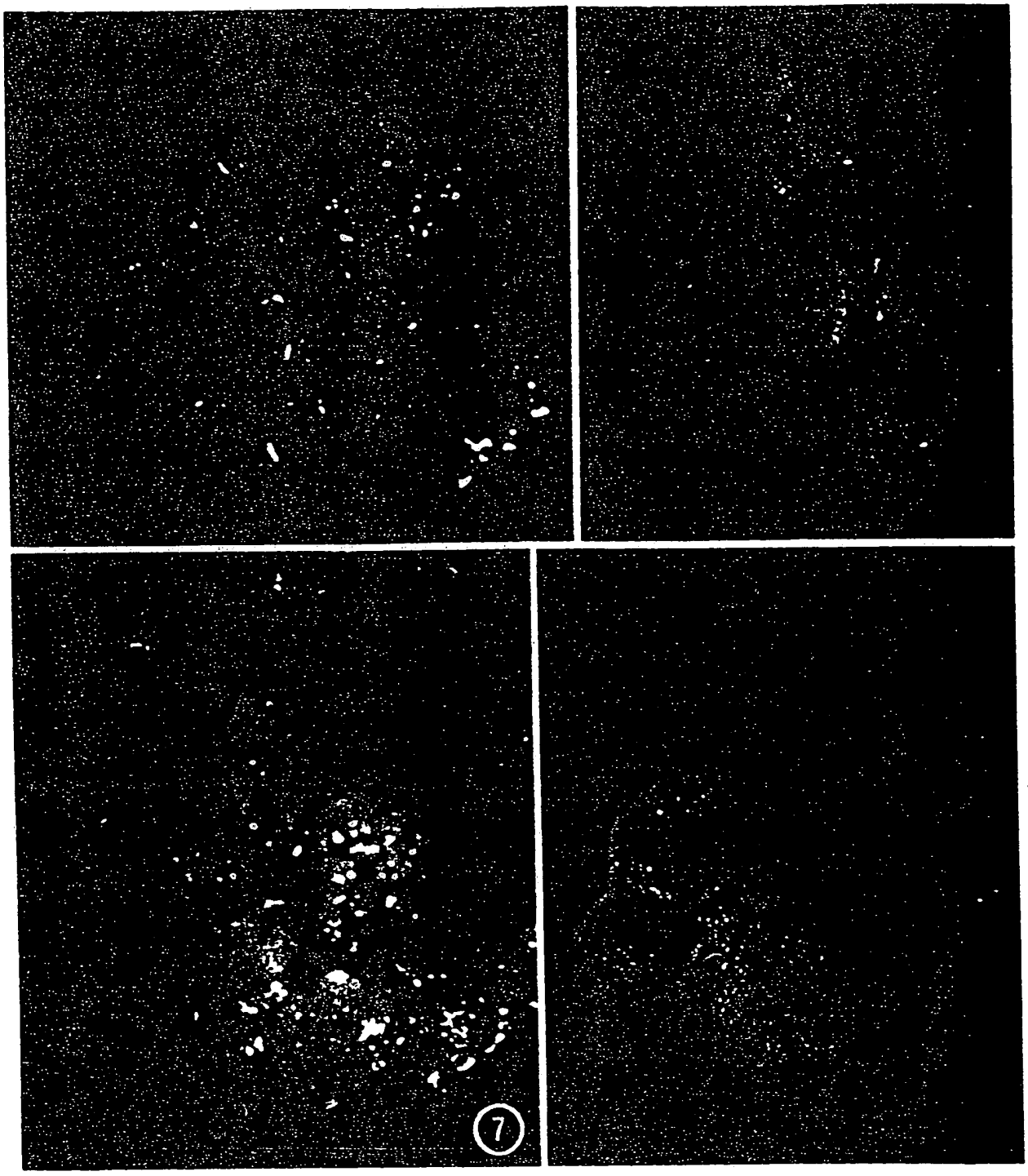


Fig. 6–9. Morphogenesis in protoplast-derived calli of *Zea mays* L. 6 and 7 Compact and organized sectors of protoplast-derived callus (×200, ×75). 8 Organised and folded structures formed in the protocalli (×540). 9 Somatic embryo with scutellum (SC) and coleoptile (CL) (×770)

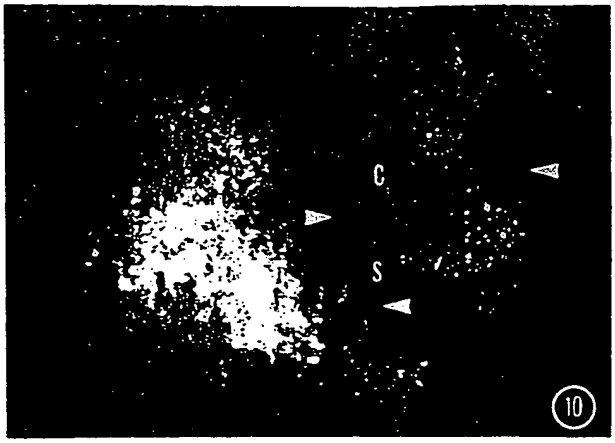


Fig. 10. A cluster (arrow heads) of precociously germinating somatic embryos (S scutellum, C coleoptile)

Growth, organization and formation of somatic embryos in protoplast-derived callus

Compact protocolonies were formed both in liquid and agarose media approximately three weeks after culture. Some of the colonies were highly organized and spherical with a distinctly uniform and continuous surface layer reminiscent of globular embryos (Fig. 5). The protocolonies continued to grow and produced a soft, beige-colored, and mucilaginous callus 3–4 weeks after transfer to agar-solidified suspension maintenance medium. The callus was unorganised initially but differentiated deep yellow to pale green sectors upon transfer to the same medium containing 0.1–0.2 mg/l 2,4-D and 0.2 mg/l ABA (Fig. 6). Parts of these sectors became further organized and somewhat white in color (Fig. 7). Leafy appendages, organised sectors with an outer epidermal layer, and highly organised folded scutellar structures (Fig. 8) developed in some of the protoplast-derived calli. Somatic embryos with an identifiable scutellum and coleoptile were formed (Fig. 9). These were not fully organised and did not reach maturity. Clusters of somatic embryos germinated precociously and parts of the scutellum became green and leafy in light (Fig. 10). An elongated green coleoptile was formed in the germinating somatic embryos.

The addition of L-proline (10–160 mM) substantially increased the growth of the callus but was not helpful in increasing the degree of organization or embryo development.

Discussion

This study has extended the success previously reported in the culture of protoplasts of 'Black Mexican Sweet' (Chourey and Zurawski 1981; Ludwig et al. 1985) to a commercial hybrid cultivar of maize.

Plating efficiency of 4–5% obtained in the present experiments, although low in comparison to the 20–30% reported in similar embryogenic protoplasts of other gramineous species (Vasil et al. 1983; Srinivasan and Vasil 1986), nevertheless compared well with earlier reports on maize (Chourey and Zurawski 1981; Ludwig et al. 1985). Further improvements can perhaps be obtained by using the cell suspension as a nurse or feeder layer.

Ludwig et al. (1985) did not find any significant variations in the callus forming ability of 'Black Mexican Sweet' protoplasts. In our experiments protoplast yields, frequency of spontaneous fusions, and the rate of division of protoplasts varied from experiment to experiment. This is similar to the results obtained in this laboratory with protoplasts isolated from embryogenic suspension cultures of several other species of the Gramineae. The presence of 2,4-D was found to be essential for the induction of division in cultured protoplasts of maize, unlike those of *Panicum maximum* (Lu et al. 1981) which divided in the absence of 2,4-D and showed a vigorous capacity for colony formation when 0.1–1 mg/l 2,4-D was added. Optimal colony formation from embryogenic protoplasts in the present study was obtained in a liquid medium containing 0.5 mg/l 2,4-D, in comparison to 2 mg/l reported for protoplasts from non-morphogenic suspensions (Chourey and Zurawski 1981; Ludwig et al. 1985).

The frequency of colony formation at 0.3% agarose was about the same as that in liquid medium. Higher concentrations of agarose inhibited colony formation. These results are consistent with our earlier experience with protoplasts of *Pennisetum purpureum* (Vasil et al. 1983).

A second and perhaps more important objective of this study, namely the establishment of a reliable protoplast culture system from a morphogenically competent cell line of maize has been also achieved. In all previous studies on the culture of maize protoplasts, only non-morphogenic cell lines were used. The suspension culture used in the present experiments, when initially established, formed somatic embryos and plants which could be grown to maturity (V Vasil and Vasil 1986). At the time of protoplast culture, about 15 months later, the cell lines were still embryogenic as evidenced by the formation of distinct somatic embryos.

The cell colonies recovered from suspension-derived protoplasts resembled the groups of embryogenic cells found in the suspension cultures as well as protocolonies of *Saccharum officinarum* (Srinivasan and Vasil 1986) from which somatic embryos and mature plants were obtained. The formation of somatic embryos in protoplast derived calli of maize in low 2,4-D media is similar to the results obtained in *Panicum maximum* (Lu et al. 1981) and *Pennisetum purpureum* (Vasil et al. 1983). Recovery of mature plants from these calli, as reported recently from embryogenic protoplasts of sugarcane (Srinivasan and Vasil 1986) and rice (Yamada et al. 1986), would be especially meaningful in light of the stable transformation of protoplasts isolated from non-morphogenic maize (Fromm et al. 1986) and embryogenic *Panicum maximum* (Hauptmann et al. unpubl. results) suspension cultures.

Acknowledgements. We gratefully acknowledge the cooperation of Dr. L. C. Hannah in growing the maize plants used in this study. This work was carried out under a research program supported by Monsanto Company, St. Louis, MO, and a collaborative project between the Gas Research Institute and the Institute of Food and Agricultural Sciences, Florida Agriculture Experiment Station Journal Series No. 7830.

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GENE TRANSFER TO CEREALS: AN ASSESSMENT

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In the following review, I present an assessment of the realities and possibilities of effecting gene transfer to cereal crops. I discuss why *Agrobacterium* has been unsuccessful with cereals, what alternatives have been tested, the extent to which they have yielded transgenic plants, and their potential agronomic utility. The discussion, necessarily subjective, is framed within a rigid definition of what constitutes proof of gene integration, and the biological factors affecting transformation competence.

The first transgenic plants expressing engineered foreign genes where recovered in 1984 (see review[1]). The five years since then have yielded such exciting results it is not surprising that numerous optimistic reviews have been written. One of the most recent[1] makes the following typical statement: "Dramatic progress has been made in the development of gene transfer systems for higher plants. . . . In view of the rapid progress that is being made, it is likely that all major dicotyledoneous and monocotyledoneous crop species will be amenable to crop improvement by genetic engineering within the next few years." Such optimism is understandable and generally good for further progress, but my personal experience in working towards the genetic engineering of cereals for the last 18 years convinces me that we still have serious problems in front of us. Success requires more than occasional gene transfer into experimentally well suited varieties of some species. It requires routine and efficient gene transfer into any desired variety of any given species, changing the genome only by the addition of one defined gene. It seems to me that we are really not yet close to such a situation. Usually gene transfer to plants is achieved with *Agrobacterium*, but as this biological vector does not function with cereals a variety of alternative approaches for gene transfer to these species have been developed, of which only one has so far been successful. Even the successful method has its problems. In the following review I will discuss why *Agrobacterium* has been unsuccessful with cereals, what alternative methods have been tested, which of those have yielded transgenic cereals, which may have a realistic chance to become a successful technique, and which may not have much potential at all. The assessment will be subjective. It will be based on a rigid definition of what constitutes *proof* of successful integrative transformation. Those who disagree with the view that indicative evidence is misleading may not agree with this assessment. The review will also be based on an interpretation of the biological factors affecting gene transfer, and several statements will be made for which no solid experimental data are available. Acceptance or refusal of these statements will not affect assessment of the available data but will only influence attitudes as to the future potential of the various approaches.

1. Proof and competence (Figure 1). If one trusts indicative evidence, there are numerous different methods to produce transgenic cereals. However, since no transgenic cereals exist except for those that have been recovered from protoplasts and direct gene transfer, there must be something wrong with the indicative evidence. Indeed many researchers have obviously been misled by artifacts and it is good advice to believe in transgenic plants only if suitable proof is available. What constitutes suitable proof? Neither genetic, phenotypic or physical data alone are acceptable. *Proof* for integrative transformation requires:

(1) Serious controls for treatments and analysis. (2) A

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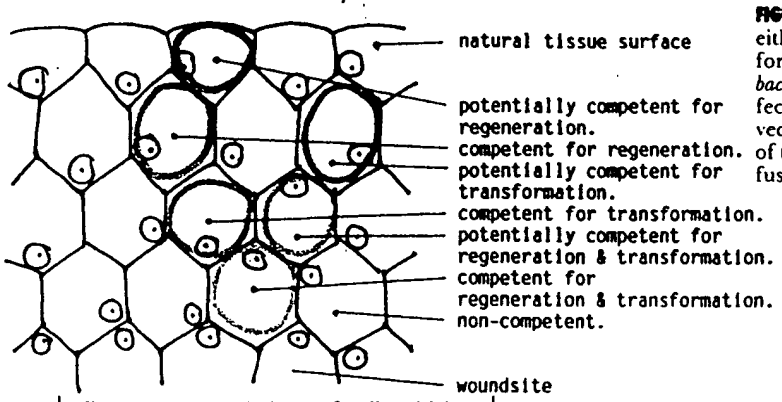
1 TISSUES ARE POPULATIONS OF CELLS NON COMPETENT, POTENTIALLY COMPETENT, AND COMPETENT REGENERATION & TRANSFORMATION.



Tissues are populations of cells which are either non-competent, potentially competent, or competent for regeneration and/or integrative transformation.

FIGURES 1–11 (1) Tissues are populations of cells which are either non-competent, potentially competent, or competent for regeneration and/or integrative transformation. (2) *Agrobacterium* and dicots. (3) *Agrobacterium* and cereals. (4) Agroinfection and dicots. (5) Agroinfection and cereals. (6) Viral vectors. (7) Incubation of dry seeds/embryos. (8) Incubation of tissues/cells in DNA. (9) Pollentube pathway. (10) Liposome fusion. (11) Liposome injection.

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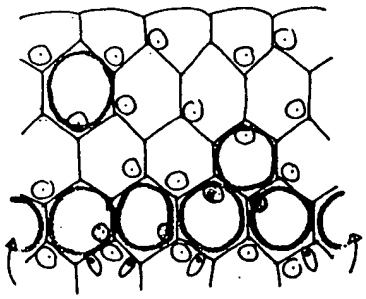

Wound response makes potentially competent cells competent

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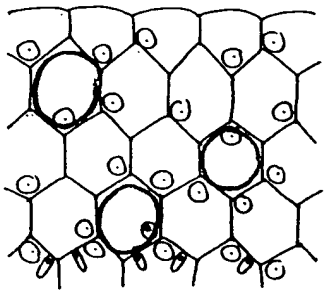

No competent cells because wound response is missing.

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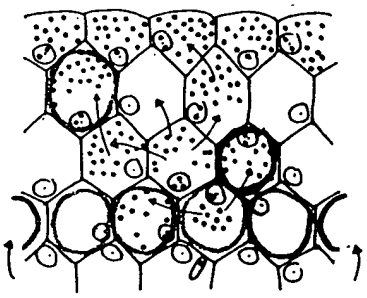

Virus spreads systemically, but does not integrate.

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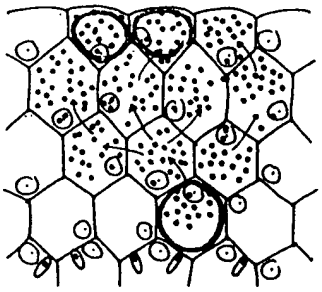

Virus spreads systemically, but does not integrate.

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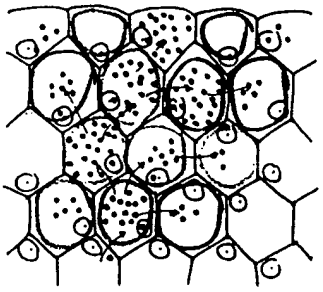

Systemic spread, no integration, no transmission to offspring.

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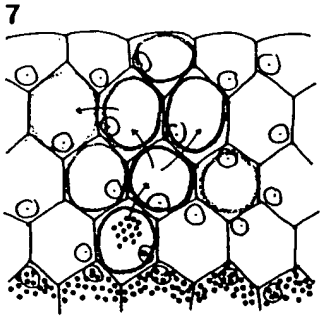
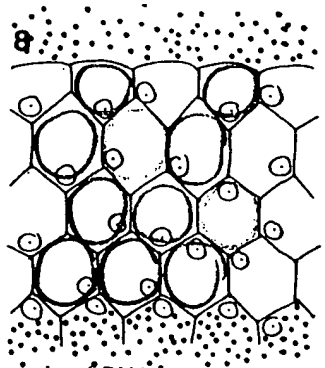


Viral DNA spreads, does not integrate. Non-viral DNA does not enter tissue.

8



No uptake of DNA across cell walls, or as very rare event only

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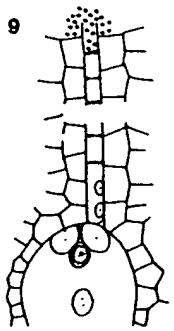

No open pipes to the egg cell; DNA adhesion to cell walls; nucleases.

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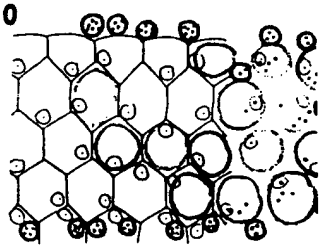
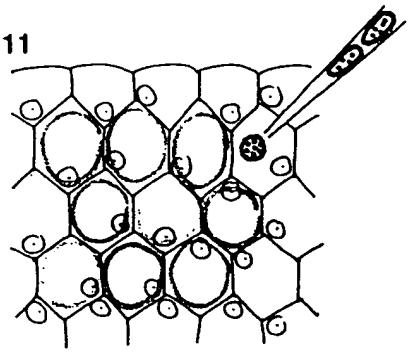


Transfers DNA into protoplasts but not into cells with cell walls.

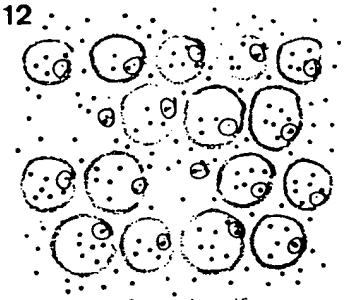
11



Transfers DNA into cells avoiding the vacuole.

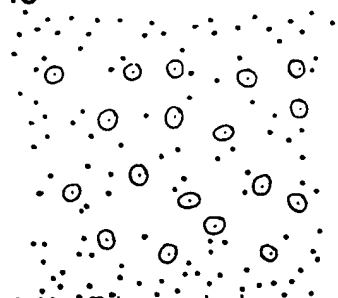
FIGURES 12–23 (12) Protoplasts and direct gene transfer. (13) Protoplasts from cereal plants. (14) Microlaser. (15) Electrophoresis into tissues. (16) Biolistics or particle gun. (17) Biolistics and cell cultures. (18) Microinjection into tissues. (19) Microinjection into proembryos. (20) *Agrobacterium* and zygotic proembryos. (21) Macroinjection. (22) Pollen incubation. (23) Electroporation.

12



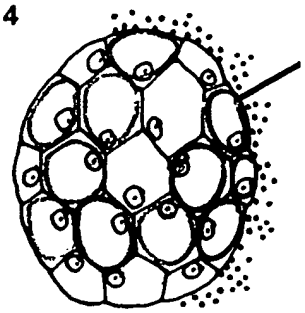
Efficient transformation if competent protoplasts available.

13



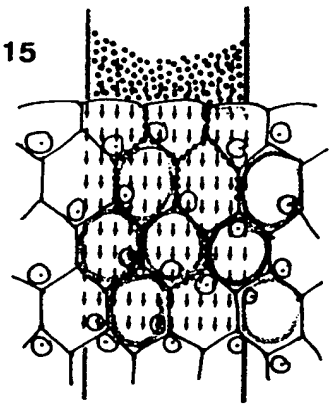
Probably efficient uptake, but no competent protoplasts.

14



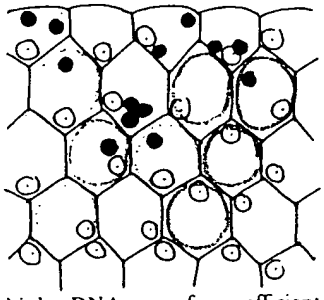
Opens holes in cell walls and membranes; DNA adhesion to cell walls.

15



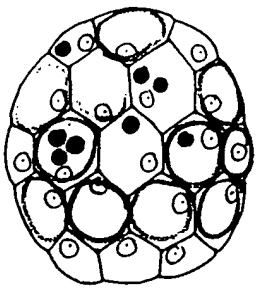
Probably no DNA transport across walls, no transgenic clones so far.

16



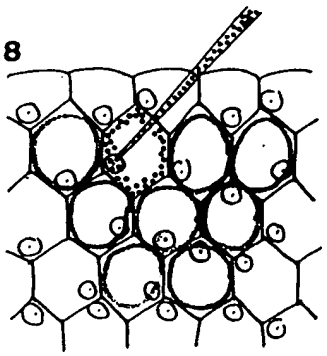
Multiple DNA transfers: efficient in transient, inefficient in integrative transformation.

17



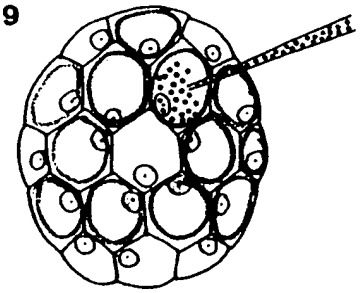
Transgenic clones at low frequency; no transgenic cereals, but promising.

18



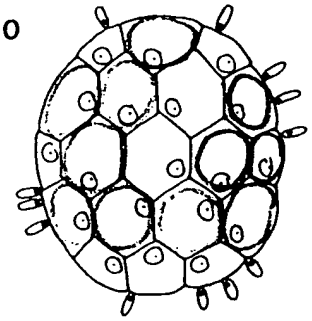
Precise delivery of DNA quantities; Transgenic clones from protoplasts.

19



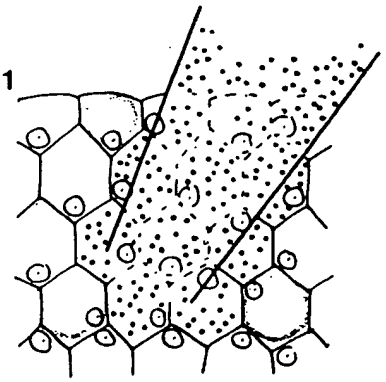
Transgenic chimeras at low frequency; no transgenic cereals, but promising.

20



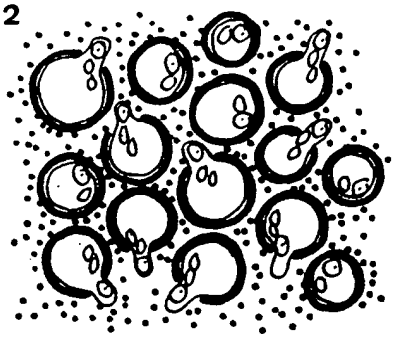
So far no transgenic tissue even in tobacco.

21



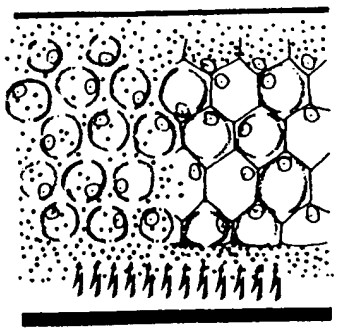
Injection destroys cells which receive DNA; no DNA transport to competent cells.

22



Probably no uptake across pollentube wall; nucleases; no integration into sperm cells.

23



Efficient DNA transfer to protoplasts. No transport across cell walls.

tight correlation between .tment and predicted results. (3) A tight correlation between physical (Southern blot, *in situ* hybridization) and phenotypic (enzyme assays) data. (4) *Complete Southern analysis* containing (a) the predicted signals in high molecular weight DNA, in hybrid fragments between host DNA and foreign gene, and the complete gene, and (b) evidence for the absence of contaminating DNA fragments or the identification of such fragments. (5) Data that allow discrimination between false positives and correct transformants in the evaluation of the phenotypic evidence. (6) Correlation of the physical and phenotypic evidence with transmission to sexual offspring, as well as genetic and molecular analysis of offspring populations.

Consideration of the *biology* of gene transfer may be helpful in understanding why some techniques work and others never do. It may also help in assessing the future potential of the various approaches. A transgenic plant can only result from integrative transformation in a totipotent cell or a cell that has a clonal connection to the "germline".

(1) Not all plant cells are totipotent. (2) Plant cells differ in their capacity to respond to triggers, a phenomenon termed *competence.*) (3) Cells from which it is hoped to regenerate transgenic plants must be competent for both regeneration (in a broad sense) and integrative transformation. (4) Plant tissues are composed of cells competent for many different responses. Considering the two states of competence essential for recovery of transgenic plants the following situation has to be considered: (a) A very small minority of cells in plant tissues will be *competent for both transformation and regeneration.* (b) Others will be competent for transformation *or* regeneration. (c) A larger fraction of the cell population will be *potentially competent,* which means that given the correct treatment they will have the potential to shift to the competent state. (d) A variable proportion of cells will not even be potentially competent but will be *non-competent.* (5) The relative composition of cell populations in tissues is determined by the genotype, the type of organ, the developmental state of the organ, and even the individual history of the experimental plant. (6) The most effective trigger for shifting potentially competent cells to the competent state is mechanical (and enzymatic) wounding. The wound response[2] is probably the biological basis of regeneration from somatic cells. (7) Plant species differ in their wound response as do different tissues of the same plant. Graminaceous plant species, especially the cereals and maize, have only a very rudimentary or no wound response. (8) For some genotypes it is possible to proliferate cells competent for regeneration under conditions that maintain this state[3]. Such cell cultures contain cells competent for regeneration and (after protoplasting?) competent for integrative transformation. (9) Plant cell walls are efficient barriers and traps for DNA molecules. (10) Genes can be transported into cells across cell walls with the help of *Agrobacterium,* "biolistics" and microinjection. (11) Production of transgenic plants requires efficient gene transfer into cells competent for regeneration and integrative transformation. (12) Competence for integrative transformation is obviously very different from competence for transient expression. (13) Non-viral DNA can integrate into the host genome. Its presence in a cell does not guarantee its integration. (14) Non-viral DNA does not move from cell to cell but is restricted to the cell to which it has been delivered. (15) Viral DNA (and RNA) moves from cell to cell and can spread systemically throughout an entire plant. It is, however, probably excluded from the meristems and the "germline". (16) Viral DNA does not integrate into the host genome even if present at very high copy nun. ..

On this basis an assessment of the various gene transfer approaches presented in the literature or discussed at international meetings is relatively easy. The cartoons help understanding by visualising the biological problems. As the background literature is extensive, I have relied to a large extent on recent reviews. I intend this to be a provocative and working hypothesis. It is, however, in agreement with all the available data from the literature and from public meetings.

2. *Agrobacterium* and dicots[4–7] (Fig. 2). *A routine and efficient method for the production of transgenic plants from numerous non-cereal species.* This includes many cases where transformation was possible only on the basis of intensive screening for the optimal combination of plant genotype and bacterial strain, or where transformation was possible only at a short developmental stage of specific tissues of the host plant, and in addition, numerous cases where transformation has so far not been possible. Interpretation: Plants and tissues differ in their wound response. Only plants and tissues with a pronounced wound response develop larger populations of wound-adjacent competent cells for efficient transformation. Dicots that have not been transformed probably do not show the appropriate wound response.

3. *Agrobacterium* and cereals[8] (Fig. 3). *No transgenic cereal recovered so far; very little potential.* Most attempts have not been published because they were negative; some promising data have been presented at international meetings but since no proof was reported, the data must be considered artifactual. Transformation of "monocots"[9] (and citations therein) are of no importance in this context as cereals are not difficult to transform because they are monocots, but because they show no wound response. Monocots with a wound response (e.g. asparagus and yam) are as easy to transform as dicots with a wound response. And dicots without a wound response are as difficult to transform as cereals. Why has it been impossible to transform cereals with *Agrobacterium*? Wounding of differentiated cereal tissue does not lead to the wound response-induced dedifferentiation in wound-adjacent cells. Therefore no competent cells are available. Instead wounding leads to the death of the wound-adjacent cells. Even though *Agrobacterium* is very efficiently at transferring its T-DNA into cereal cells, (see section 5. *Agroinfection and cereals*) integration of this T-DNA can not lead to transgenic clones because the receptor cells die. It is not as easy to understand why even in those experiments where meristematic tissue (e.g. leaf base or split shoot tip), which can be induced to form proliferating cultures and plants, did not yield transgenic plants. One possibility is that cereal cell cultures are not the consequence of proliferating wound meristems but are based on adventitious meristems[10]. Wounding plus *in vitro* culture does not lead to many competent cells but to a few meristem initials that proliferate as meristems. Meristematic cells may not be transformation competent (see section 17. *Microinjection* and section 18. *Agrobacterium and zygotic proembryos*).

Raineri et al.[54] presented three different lines of evidence giving a reasonable inference of *Agrobacterium*-mediated transformation of rice (*Oryza Sativa*). Although definitive proof, as defined within the present context, is still lacking, it should be relatively straightforward to obtain the necessary data.

4. Agroinfection and dicots[11,12] (Fig. 4). *Agroinfection can lead to transgenic plants via T-DNA integration.* Viral DNA integrated into the T-DNA of the Ti-plasmid of *Agrobacterium* can be delivered into plant cells with the normal T-DNA transfer process. The consequences of agroinfection in dicots with a wound response are the

following: The virus enters the cell as part of the T-DNA. It is released to form a functional virus that replicates and spreads systematically. It may not be necessary for the T-DNA to integrate in order to release the virus. Systemic spread of the virus as a consequence of agroinfection is, therefore, no proof of integration of foreign DNA. T-DNA *can*, however, integrate and thus agroinfection can lead to integration of viral DNA in the wound-adjacent cells and consequently to transgenic plants containing integrated viral DNA. This is not different from normal T-DNA transformation. The important point is that there is no integration of the systemically spreading virus. Can agroinfection be used to transform cereals?

5. Agroinfection and cereals[13] (Fig. 5). *This method has very little potential for the production of transgenic cereals.* Agroinfection has been shown to lead to systemic spread of maize streak virus. This showed for the first time that *Agrobacterium* can transfer its T-DNA to cereal cells. Later it was demonstrated that the efficiency of transfer is comparable to dicot systems. Does this method, therefore, have potential for the production of transgenic cereals? In cereals, agroinfection leads to the transfer of the virus-carrying T-DNA into wound-adjacent cells. The virus is released, replicates and spreads systemically. If it reaches rare competent cells somewhere in the plant body it will not integrate. At the woundsite, the T-DNA faces the same problems as discussed in section 3. Thus the chances that agroinfection will produce transgenic cereals are minimal and no different normal *Agrobacterium* infection—unless somebody finds a way to induce integration of viral DNA, or of foreign DNA integrated into a replicating and spreading virus, as could be envisaged for a transposable element carried through the plant by a spreading virus.

6. Viral vectors[14–17] (Fig. 6). *This method has very little potential for production of transgenic cereals, although it is very interesting for amplification of genes and gene products.* Viruses spread systemically throughout the plant from the infection site and can replicate many thousands of copies per cell. The discovery that RNA virus genomes can be reverse transcribed to yield cDNA clones that again are infective opened the possibility of using genetic engineering technology not only with the relatively small group of DNA viruses but also with the larger group of RNA viruses. According to the available evidence, viruses do not integrate into the host genome and they are excluded from meristems and thus from transmission to sexual offspring. These facts determine the advantages and disadvantages of viruses as vectors for possible genetic engineering in plants. Although it would be very difficult (except e.g. via integrated transposable elements) to use viral vectors for integrative transformation, they have invaluable potential for gene amplification and systemic spread within individual plants.

7. Incubation in DNA of dry seeds or embryos[18–21] (Fig. 7). *Thus far, no transgenic plants have been recovered, not much potential.* Incubation of seeds in DNA has yielded indicative evidence since the 1960's. However, no proof for integrative transformation has ever been presented. Töpfer et al.[19] describe experiments in which every precaution was taken to avoid the experimental pitfalls of earlier experiments and these yielded very interesting evidence for transformation following incubation of dry seeds or embryos in engineered viral and non-viral DNA. Although the experiments contain convincing controls and clearly demonstrate the presence and expression of defined marker genes as well as the replication of engineered viral DNA, they do not provide proof for integrative transformation. Their conclusion that the data demonstrate uptake of the foreign DNA into the cells of the embryos is really one hypothesis. It is, to date not possible to exclude the alternative hypothesis that the DNA data are the result of transient reactions in the micro-environment of open cells at the large wound site and that the virus DNA data are, in addition, due to systemic spread. Only the analysis of offspring from regenerated plants will finally show which hypothesis is correct. So far, I favour the following interpretation: If engineered viral DNA is used, it may spread systemically and even reach competent cells; it will not integrate. If non-viral DNA is used, it will most probably not even be taken up into the wound-adjacent cell layer or only at very low efficiency. Wound-adjacent cells will not include competent cells or they will be present only at very low frequency, and these would not be induced to proliferate to form cell clones. The DNA would not reach the meristematic cells of the shoot apex because it would have to travel across numerous cell walls. If the treatment were to be modified to allow direct access to the shoot meristem, regeneration would not occur from the wounded shoot apex but from non-wounded axillary meristems of the leaves. Therefore regeneration of transgenic plants is highly improbable.

8. Incubation in DNA of tissues or cells (Fig. 8). *No transgenic tissues or plants have been recovered; very low potential.* There have been many approaches where seedlings, organs, tissues, cells, or cell cultures of numerous plant species have been brought into direct contact with foreign DNA and defined marker genes. Treatments also included experimental designs making use of open plasmodesmata or loosening of cell wall structures. There were also treatments ensuring that competent cells were available at a sufficient frequency. Even in experiments that would have recovered extremely rare events of integrative transformation, there is not a single proven case of integrative transformation. Experiments relying on the passage of functional genes across cell walls have very little chance of success, not only because the cell wall is a perfect barrier to large DNA molecules, but because it is also an efficient trap. Even if there were occasional transfer there are other negative parameters that act in a multiplicative way: (a) attachment to cell walls, (b) transport across further cell walls, (c) no mechanism for DNA transport, (d) competent cells have to be reached. The combination of several low frequency events will cause problems even if one step may occasionally work.

9. Pollentube pathway[22] (Fig. 9). *No transgenic plants have been recovered; probably not much potential.* If it were possible to deliver DNA to the zygote via open pollen tubes in the course of normal pollination, this would be very attractive. Unfortunately, the recent publication presenting molecular data on transgenic rice plants does not present proof. The Southern data do not show integration into high molecular weight DNA and defined hybrid fragments, and can be easily interpreted as artifacts: the dot blot technique is prone to artifacts and the enzyme data are not reliable because cereals have a rich record of false positives with the assay used. It is also difficult to envisage how this system *should* work: the pollen tubes are not open pipes but sealed off with callose plugs: the DNA will be trapped by the cell wall material, there are probably nucleases not only in the synergids but also in the pollen tube; there is no transport system known. Because of the attractiveness of this approach it is still worthy of rigorous testing.

10. Liposome fusion with tissues and protoplasts[23–25] (Fig. 10). *Transgenic plants have been recovered from protoplasts but not from tissues and cells; very little potential with cells.* Fusion of DNA-containing liposomes with protoplasts is an established method for the production of transgenic plants. It has, however, no obvious advantage over direct

gene transfer (see section 12) and electroporation (see section 21). DNA-containing liposomes have also been applied to various tissues, cell cultures and pollen tubes, with the rationale that liposomes might help transport the DNA via plasmodesmata or directly across the cell wall. It has been shown that liposomes can carry small dye molecules into cells within tissues via fusion with the plasmalemma. There is, however, no proof for transport and integration of marker genes. As plasmodesmata are sealed off immediately upon wounding this route is not open even for small liposomes; impregnation of the cell wall with phospholipids does not seem to change its barrier function.

11. Liposome injection[26] (Fig. 11). *Thus far no transgenic tissue recovered.* Microinjection of DNA has yielded transgenic chimeras (see section 17). Microinjection into differentiated cells can easily deposite the DNA into the vacuole, where it is degraded. W. Lucas et al.[26] had the elegant idea to exploit the vacuole. Microinjection of liposomes into the vacuole leads to fusion with the tonoplast thus releasing the content of the liposome into the cytoplasm, as demonstrated with cytoplasm-activated fluorescent dyes. Activity of injected DNA has still to be shown. This method, though elegant, has probably no advantage over straight forward microinjection especially for the production of transgenic cereals. Cereals regenerate only from meristematic cells that do not have large vacuoles.

12. Protoplasts and direct gene transfer[27–35] (Fig. 12). *The only method that has thus far yielded transgenic cereals; still problematic because plant regeneration from protoplasts is difficult to achieve.* Protoplasts efficiently take up DNA if treated with polyethyleneglycol (PEG) and/or electroporation. The protoplast isolation procedure probably shifts potentially competent cells to the competent state. If protoplast populations are available that contain competent cells, exogenous DNA is easily integrated via nonhomologous recombination. Also homologous recombination occurs, but at a far lower frequency. When protoplasts are transformed that are also competent for regeneration, transgenic plants can be recovered that stably contain, express and inherit the foreign gene. Protoplasts isolated from cereal tissues do not contain cells competent for regeneration (see section 13). Competent protoplasts have, so far, been isolated only from embryogenic suspensions established from immature tissues (scutellum, leaf base, anther). Standard direct gene transfer procedures with protoplasts from embryogenic suspensions has led to the regeneration of transgenic rice (*Oryza sativa* var japonica and *indica*) and maize (*Zea mays*) and will yield transgenic plants from other cereal species as soon as routine and efficient plant regeneration from protoplasts is established. This is, however, likely to be a problem for some years because, so far, the establishment of the appropriate cell cultures is an art that also depends upon parameters beyond experimental control. It is, therefore, difficult to envisage that this approach will ever serve as a solid basis for the required routine and efficient procedure for gene transfer into any desired species and variety.

13. Protoplasts from cereal plants[36] (Fig. 13). *No transgenic clones have been recovered; no potential to date.* As the establishment of appropriate embryogenic suspensions is a delicate and often unpredictable process, it would be of great advantage if protoplasts isolated directly from differentiated tissues could be cultured. However, this approach appears, to date, rather hopeless because differentiated cereal tissues do not express the wound response and obviously do not contain cells competent for regeneration. DNA uptake is no problem, as can be shown easily with transient expression assays. If integration occurs it has no consequences, because protoplasts do not proliferate. Although intensive experimentation has failed, so far, to induce proliferation, I would encourage further attempts.

14. Microlaser[37] (Fig. 14). *No transgenic tissue produced; not much potential.* A microlaser beam focussed into the light path of a microscope can be used to burn holes into cell wall and membranes. It was hoped that incubation of perforated cells in DNA solutions could serve as a basis for a vector-independent gene transfer method into walled plant cells. There are no conclusive data available on DNA uptake and there are problems with adsorption of exogenous DNA to cell wall material, even before it could be taken up. As microinjection and biolistics definitely transfers DNA into walled plant cells (see sections 16 and 17), the microlaser would offer advantages only in very specific cases where those techniques were not applicable.

15. Electrophoresis into tissues[38] (Fig. 15). *There is no proof for integrative transformation; judgement of the potential requires further experimentation.* Ahokas[38] tried to electrophorese DNA across the shoot meristem of barley seeds. His experiments yielded indicative evidence in the form of radioactively-labeled cell walls, positive GUS assays, and a protein on SDS-PAGE with *E. coli* GUS mobility. So far, all the data can also be interpreted as artifacts. It might, however, be worthwhile to test the potential of the idea with an experimental system that can give clearcut answers.

16. Biolistics or particle gun[39–42] (Figs. 16 and 17). *Thus far, no transgenic offspring produced in cereals. The method does, however, have good potential and is excellent for testing gene expression in transient systems.* Acceleration of heavy particles covered with DNA can be used to transport genes into plant cells and tissues. This technique caused some excitement because it was believed for some time that it would solve all gene transfer problems. Since then it has become possible to discuss "biolistics" on a more realistic basis. Transgenic plants have been produced in soybean and tobacco, and others will follow. The method has advantages that do give it the potential for general applicability: (1) it is easy to handle; (2) one shot can lead to multiple hits; (3) cells survive the intrusion of (one?) particle; (4) the genes coated onto the particle resume biological activity; (5) target cells can be as different as pollen, cell cultures, plant organs, and meristems; (6) particles also reach deeper cell layers. Thus the method provides a biological vector-independent DNA delivery system into a great variety of cells. Why then with all these advantages have no transgenic cereals been produced? As numerous particle gun machines are in use in many research groups, and many scientists have been shooting marker genes into many different experimental materials, we must assume that there are inherent problems. One of the technical problems for which improvement can be foreseen is the low frequency of transient and integrative events. As long as transient events occur at frequencies of ca. 10^{-4} and integrative events at ca. 10^{-8}, large numbers must be produced to hit one of the rare competent cells. Great effort and careful optimization of the parameters of the technique seem to improve the situation considerably. There remains the problem that the particles have to hit competent cells, which are very rare in cereals as long as embryogenic cell cultures are not available. As protoplasts isolated from embryogenic cell cultures definitely contain competent cells, and as these protoplasts are relatively easy to transform, direct gene transfer into protoplasts should be the method of choice

for those cases where such cultures have been established. The route via shoot meristems (in analogy to the successful soybean case) is considerably more difficult for cereals, because (a) the meristem is far better protected and more difficult to expose, (b) regeneration via multiple adventitious shoots from the tissue below the meristem is far less efficient, and (c) it is still an open question whether biolistics can transform meristematic cells. This is not unimportant for cereals because in cereals regeneration requires meristematic cells.

17. Microinjection[43,44] (Figs. 18 and 19). *No transgenic offspring have been recovered so far in cereals. The method does, however, have good potential.* Microinjection uses microcapillaries and microscopic devices to deliver DNA into defined cells in such a way that the injected cell survives and can proliferate. This technique has produced transgenic clones from protoplasts (where transformation via direct gene transfer is easier) and transgenic chimeras from microspore-derived proembryos in oilseed rape. As with biolistics, microinjection definitely delivers DNA into cells. In comparison with biolistics, microinjection has disadvantages (only one cell receives DNA per injection; the handling requires more skill and instrumentation). It also has advantages: (1) The quantity of DNA delivered can be optimised; (2) the experimenter can decide into which cell to deliver the DNA; (3) delivery is precise and predictable, even into the cell nucleus, and is under visual control; (4) small structures (e.g. microspores and few-celled proembryos, which are not available in the large quantities required for a biolistic experiment, can be targeted; (5) defined microinjected units can be micro-cultured; (6) if zygotic proembryos can be regenerated, microinjection would offer an approach to transformation that would be open for every species and variety having sexual propagation. *If competent cells could be visually identified*, no other technique could compete with microinjection. On the assumption that few-celled zygotic proembryos contain competent cells our group has established plant regeneration from isolated zygotic proembryos of maize *(Zea mays)*, wheat *(Triticum aestivum)*, rice *(Oryza sativa)*, barley *(Hordeum vulgare)*, soybean *(Glycin max)*, cotton (*Gossypium* hybrid), sunflower *(Helianthus annuus)*, tobacco *(Nicotiana tabacum)*, and *Arabidpopsis*. Following multiple microinjections with marker genes, putative primary transgenic chimeras and sexual offspring have been analysed (G. Neuhaus, G. Spangenberg, S. K. Datta. pers.comm.). So far, we have indicative evidence for putative transgenic chimeras. As we have no proof yet for transgenic offspring, these may be artifacts. Gene transfer into structures consisting of more than one cell can only produce transgenic chimeras (independent of the technique used), and transgenic offspring can only be expected if the transgenic sector contributes to the floral meristems. Therefore, two interpretations are possible to date (as for biolistics): (a) larger experiments will increase the chance for transmission to the offspring, or (b) meristematic cells are not competent for integrative transformation.

18. Zygotic proembryos and Agrobacterium (Fig. 20). To test the second hypothesis ((b) above) an experiment has been performed using a well-established and independent gene transfer system: pre-induced *Agrobacterium* carrying a proven resistance gene (35S-NPTII-35S) was used in an attempt to infect zygotic proembryos of *Nicotiana tabacum* var SR1 (a well-documented host plant for *Agrobacterium*-mediated gene transfer). The fact that transgenic tissue could not be detected, either in the regenerated tobacco plants or in the sexual offspring, unfortunately adds some weight to the second hypothesis (G. Neuhaus, A. Matzke, M. Matzke; pers. comm.).

19. Macroinjection[45] (Fig. 21). *There is no proof for the recovery of transgenic plants; probably no potential.* Use of injection needles with diameters greater than cell diameters leads to destruction of those cells into which DNA is delivered. DNA integration would require that the DNA moves into wound-adjacent cells and, therefore, all problems discussed in sections 7 and 8 apply. The most exciting data so far were reported in an experiment where a marker gene was injected into the stem below the floral meristem of rye *(Secale cerealse)*. Hybridization to the marker gene and enzyme assays with selected sexual offspring yielded strong indicative evidence. Unfortunately, it has so far not been possible either to reproduce these data in several large-scale experiments with other cereals or to establish proof with the original material. It would be very difficult to understand how the DNA could reach the sporogenic cells in this experimental design, as DNA would not only have to reach neighbouring cells but would have to travel across many layers of cells.

20. Pollen transformation[46–49] (Fig. 22). *No transgenic plants have been produced; probably no potential.* This approach goes back to the early seventies and is based on the hope that DNA could be taken up into germinating pollen and either integrate into the sperm nucleus or reach the zygote with the pollen tube. Indeed, if this would function, this would be the ideal method for gene transfer into plants. Pollination with pollen germinated in the presence of DNA has yielded surprising results that could be interpreted as indicative evidence for gene transfer. In no case, however, has proof been provided nor could the transfer of a phenotype be shown to be caused by the transfer of a corresponding gene. As numerous large-scale experiments in experienced laboratories with defined marker genes have only given clearly negative results, it seems justified to conclude that this approach is not a very promising one. It is also understandable as not only the cell wall presents a problem, but also external and internal nucleases. The latter problem may be overcome with the technique of *in vitro* maturation[50] where immature microspores are treated with DNA, matured to pollen and then used for pollination, but here again there is no proof yet for transformation.

21. Electroporation[50–52] (Fig. 23). *No transgenic clones have been produced when applied to cells and tissues; not much potential with walled cells; routine method for gene transfer to protoplasts.* Protoplasts can be transformed with polyethyleneglycol (PEG), PEG + electroporation, electroporation alone, microinjection, and *Agrobacterium*. For protoplast systems, electroporation is but one of several modifications of direct gene transfer. Since in numerous important cases plants can be regenerated from cell cultures and tissue explants but not from protoplasts, it has been important to test whether electroporation can transfer genes into walled cells. This does not appear to be the case.

SUMMARY

Of numerous approaches to cereal transformation, so far only direct gene transfer into protoplasts has been successful. Considering the biological parameters outlined in section 1, it is not surprising that most approaches have not worked, and probably can not work. Biolistics, microinjection and *Agrobacterium* (?) have potential for a breakthrough. The fact, however, that careful and large-scale experiments with biolistic devices and microinjection into meristems and microspore-derived, zygotic and somatic proembryos have not yet yielded proof for the recovery of transgenic offspring may point to a biological problem that has not been considered in previous experiments, and which may also be the cause for the failure of

Agrobacterium-mediated transformation. Accumulated experience of gene transfer experiments with plants is in agreement with the hypothesis that meristematic (embryonic) cells can not be transformed. I do not know of any experiment that would disprove this hypothesis. It is, therefore, an important challenge for those interested in gene transfer to cereals to find out whether this is true, and if so, what prevents integrative transformation in these cells. Answers to these questions will be important independent of whether biolistics, microinjection, or *Agrobacterium* is used for gene transfer. It will, probably, be crucial for cereal transformation to solve this problem because it is not easy to regenerate cereals from non-meristematic cells. Although transgenic cereals can be regenerated from protoplasts in rice, and one has reason to hope that this will also be possible from other cereals, it would be unfortunate if gene technology with cereals has to rely on this tedious, unpredictable and unreliable method. If we can solve the problem of integrative transformation in zygotic proembryos, we can hope to have a method that can transfer genes into every variety of every plant species.

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Note added in proof: We have recently established what we believe is proof of the recovery of transgenic offspring of *Indica*-type rice. (Datta, S. K., Peterharns, A., Datta, K., Potrykus, I., 1990. Bio/Technology, submitted.

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